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<b>13. ABSTRACT (Maximum 200 Words)</b>  Rab proteins are small GTPases that are essential elements of the protein transport machinery of eukaryotic cells. Each round of membrane transport requires a cycle of Rab protein nucleotide binding and hydrolysis. My research project consists in the study of Rab GTPases, the way in which they regulate intracellular transport, and the elucidation of mechanisms by which proteins involved in intracellular protein trafficking are linked to uncontrolled cellular proliferation and cancer. Our laboratory has extensively characterized Yiplp and Yoplp, membrane proteins which appear to play a role in Rab-mediated membrane transport in <i>Saccharomyces cerevisiae</i> . This past year I have further characterized other Rab interacting membrane proteins that are homologs of Yiplp. I have also cloned the mammalian homolog of Yiplp and initiated its characterization in mammalian cells. I will present exciting new evidence on the important role of prenylation in Rab protein localization and function and a potential role for Yiplp as a specific factor that binds the di-geranylgeranylated Rab. The results will be discussed in terms of the relevance to treatment for human cancers.				
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Calero,M., Winand, N.J. and Collins,R.N.(2002) "Identification of the novel proteins Yip4p and Yip5p as Rab GTPase interacting factors". *FEBS Lett.* **515**(1-3):89-98.

## **INTRODUCTION:**

During the development and progression of human cancer, cells undergo numerous changes in morphology, proliferation and transcription. Some of the molecular mechanisms involved in these changes have involved the members of the Ras superfamily of small GTPases[1]. Rab proteins constitute the largest group of this superfamily. They are essential elements of the protein transport machinery of eukaryotic cells. Each round of membrane transport requires a cycle of Rab protein nucleotide binding and hydrolysis [2]. It is becoming more apparent that Rab GTPases, by regulating intracellular transport and events such as regulated exocytosis (in mammary gland for example) play a crucial part in uncontrolled cellular proliferation and cancer[3]. My research project aims to examine the mechanisms by which Rab GTPases regulate intracellular transport, and the elucidation of the links connecting intracellular protein traffic to uncontrolled cellular proliferation and cancer. In my first year, I reported the extensive characterization of a Rab interacting membrane protein Yop1p, the yeast homolog of the familial adenomatous polyposis locus gene known as TB2[4]. We reported that overexpression of Yop1p resulted in the accumulation of internal vesicles and cell death. Furthermore, we showed that Yop1p complexes with Yip1p, a membrane protein in yeast essential for membrane transport.

In this past year my focus has been on understanding the role of Yip1p and Yop1p in Rab function and to begin the characterization of the interactions of Rab proteins with the mammalian homologs of Yip1p. During the progress of my project I made an exciting finding that could have important implications in cancer treatment. A characteristic feature of Rab proteins is their steady state localization to the cytosolic surface of a particular subcellular membrane. Rabs stably associate with membranes by virtue of their post-translational modification; the addition of two C-20 lipid moieties (geranylgeranyl groups) onto conserved C-terminal cysteines of the protein. Much like Ras proteins, the prenyl modification functionally activates Rab proteins and enhances the propensity for membrane association. The farnesylation of p21Ras has been subject of intense research since this reaction has been shown to be essential for malignant transformation, however it is becoming more apparent that geranylgeranylated proteins have an important role in cellular proliferation [5]

I have investigated the effect of different lipid modifications on Rab protein localization and function. My data suggests that singly prenylated Rabs do not localize to the correct subcellular membrane. Our findings suggest that the two geranylgeranyl groups are not merely passive membrane anchors but rather have a crucial role in Rab protein targeting and function. Excitingly, Yip1p is a factor that specifically interacts with digeranylgeranylated Rabs. This finding has implications in the treatment of cancer. Farnesyltransferase inhibitors are promising substances in anticancer chemotherapy. It has been recently determined that these drugs have effects on both farnesylated and geranylgeranylated moieties [6]. In this scenario, the modification of Rab proteins would be impaired and this may result in undesirable side effects in treatment with Farnesyl transferase inhibitors.

## **BODY:**

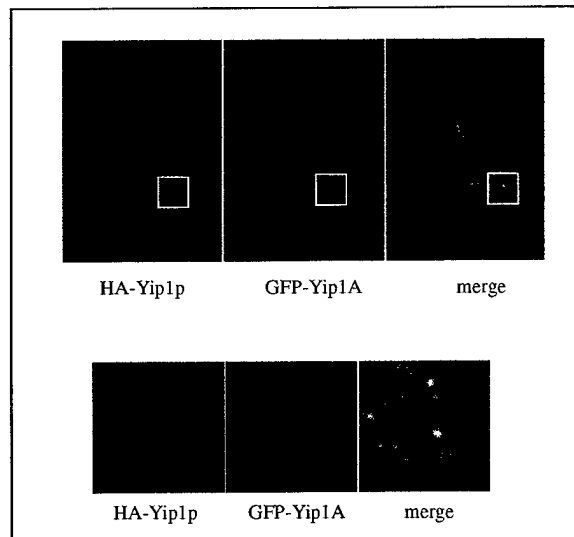
An objective in my statement of work was to focus on possible interactions of TB2 with Rab3A, as the differentiation of mammary epithelial cells into secretory lobular lveoli, promotes Rab3A expression and the accumulation of Rab3A associated vesicles in MCF7 cells [3]. Since Rat PRA1 was originally isolated as a Rab3A interacting protein, I decided to test for specificity of the interactions between the yeast homolog of Pra1 (Yip3p) and Rabs, Yip1p and Yop1p. The results of these experiments resulted in a publication in Biochemical and Biophysical Research Communications [7]. Briefly, we discovered that various Rab proteins including the yeast homolog of Rab3A (Sec4p) interact with Yip3p in a prenylation dependent manner. Furthermore, we were able to establish that Yip1p forms complexes with Yip3p.

Another objective in my statement of work was to examine homologs of Yip1p. This project was initiated by a database screen to identify sequence related Yip1p proteins. The results of this screen were ambiguous due to the small size and hydrophobic nature of Yip1p making identification of homologs difficult. We decided to examine potential primary amino acid sequence-related proteins too establish common features and to determine if we could identify a Yip1p-family. I started by cloning and characterizing two yeast homologs of Yip1p and their potential interactions with Rabs. The results of these studies resulted in a publication in FEBS Letters [8]. In this manuscript we demonstrate that the Yip1p-related proteins possess a common domain topology, are capable of biochemical interactions with a variety of Rab proteins and share an ability to physically associate with each other.

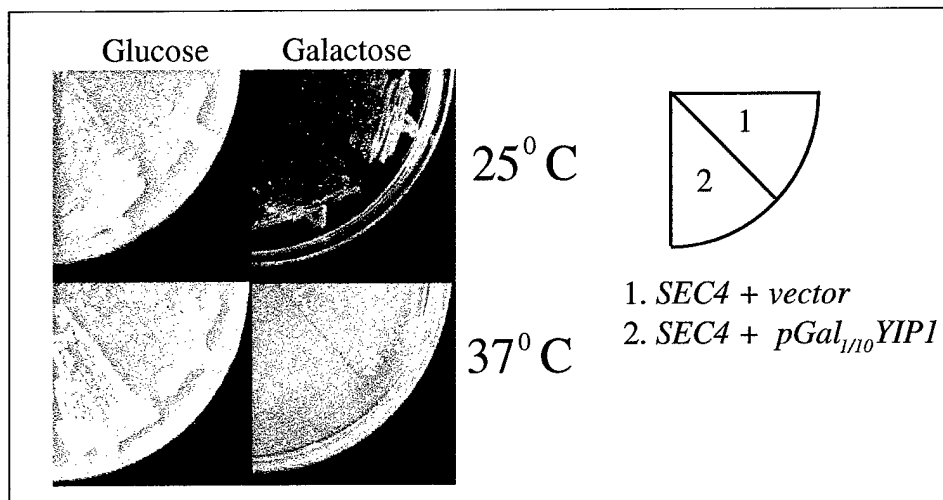
The YIP1-related proteins are found across eukaryotes. *YIP1* has at least 3 homologs in mammalian cells. I have initiated the characterization of the mammalian homolog of Yip1p, Yip1A. I have tested it for potential interactions with both mammalian and yeast Rab protein. The results of these experiments are shown in Table 1. Yip1A interacts with several different mammalian Rabs including Rab1 and Rab5. Similarly, Yip1A interacts with all of the yeast Rabs tested. Yip1p was also found to associate with mammalian Rabs Rab1A and Rab5. These results suggest the associations between Rabs and Yip are conserved throughout evolution. Further evidence of this was shown by studying the localization of Yip1p in mammalian cells. I cloned both the yeast and the mammalian Yip in a mammalian expression vector. The yeast Yip, had an HA epitope and the mammalian Yip1A a GFP tag. I transfected these constructs into HeLA cells and detected their localization by immunofluorescence. The results are shown in Figure 1. The two proteins colocalize to the same punctuated structures in the cells. The fluorescent pattern seems to reveal endoplasmic reticulum staining. These results are in agreement with recent results suggesting that Yip1A localizes in ER exit sites in the cell [9].

BAIT	PREY	RESULT
Rab1	Rab-GDI	++++
Rab1	hYIP1A	++
Rab1	yYIP	++
Rab1	Rho-GDI	-
Rab5A	Rab-GDI	+++
Rab5A	hYIP1A	++
Rab5A	yYIP1	++
Rab5A	Rho-GDI	-
SEC4	hYIP1A	+++
YPT11	hYIP1A	++
YPT6	hYIP1A	+++
YPT7	hYIP1A	+++
YPT52	hYIP1A	+++
VPS21	hYIP1A	+++
YPT1	hYIP1A	+++
YPT31	hYIP1A	++

**Table 1. Two hybrid interactions of mammalian and yeast Rab proteins with mammalian Yip1A and yeast Yip1p.** b-galactosidase activity was determined by filter assay. Pairs of constructs were coexpressed in the reporter strain Y190. Plus represents a positive activity rated according to the following criteria (++) activity detected after 30 min, (++) activity detected after 90 min, and (+) activity detected after 5 hours, and minus (-) a negative indication of activity. At least 30 independent transformants were tested for each pair.



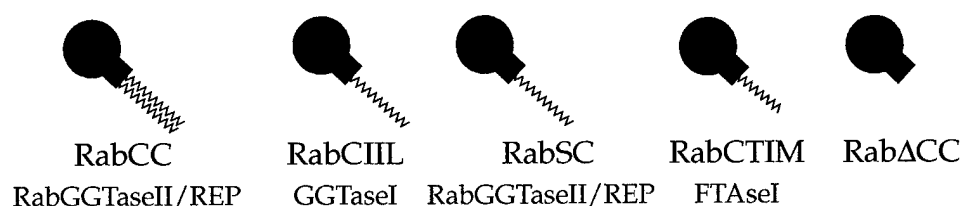
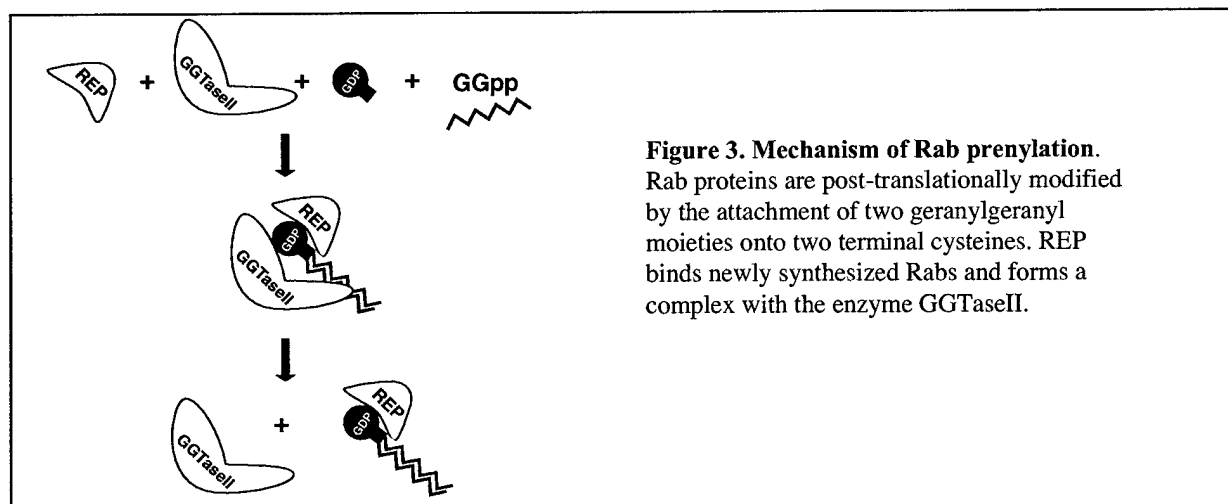
**Figure 1. Mammalian Yip1A and yeast Yip1p colocalize in HeLa cells.** Yip1A and Yip1p were cloned in mammalian expression vectors and transfected into HeLa cells. Cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, quenched with 50 mM NH<sub>4</sub>Cl-PBS, and permeabilized for 5 min with 0.1% Triton X-100-PBS. After being blocked in 10% goat serum, cells were incubated with primary and secondary antibodies for 30 min each and mounted in Moviol. HA-Yip1A was detected using a monoclonal antibody (12CA5). GFP-Yip1p was detected using the polyclonal serum kindly supplied by Pam Silver (Harvard). As secondary antibodies, we used Oregon Green-labeled goat anti-mouse immunoglobulin G (IgG) Texas Red-labeled goat anti-rabbit and goat anti-mouse IgG (Molecular Probes). Cells were viewed using a Zeiss Axioplan 2 fluorescence microscope fitted with a 40X~ or 63X~ objective lens.



**Figure 2. Yip1p overexpression renders Sec4p not functional.** Yeast strain RCY248 (*sec4-8*) is a temperature sensitive strain of Sec4p that grows at 25°C but not at 37°C. This strain was transformed with an episomal copy of Sec4p and either vector only or pRC1775 (plasmid that contains Yip1p under the control of the Gal <sub>1/10</sub> promoter). Yip1p is overexpressed in Galactose but repress under glucose containing media. On Galactose, when Yip1p is overexpressed *SEC4* cannot rescue the temperature sensitive strain.

One of the outstanding questions in my research is what is the function of Yip1p and Yop1p. I have new data to suggest that Yip1p is a negative regulator of Sec4p. This experiment made use of a plasmid containing Yip1p under the control of the Gal <sub>1/10</sub> promoter in order to induce the overexpression of Yip1p in galactose containing media but repress the expression in glucose containing media. I transformed this construct in a temperature sensitive strain of Sec4p (*sec4-8*) that grows at 25°C but not at 37°C together with a WT copy of *SEC4* on an episomal plasmid. As shown in Figure 2, under glucose, *SEC4* rescues the temperature sensitivity as the cells are able to grow. However, under galactose, *SEC4* is not able to suppress the temperature sensitivity and the cells die. The control strain without the GAL<sub>1/10</sub>-Yip1p plasmid is able to rescue growth. This is an *in vivo* indication that Yip1p plays an important role in the function of SEC4 and could potentially be a negative regulator of this Rab. It will be interesting to determine the role of Yop1p in this pathway and to then investigate the effects of overexpression of Yip1p and Yop1p mammalian homologs in MCF7 cells. I plan to conduct such experiments in my final year of funding.

During the progress of my work, while trying to investigate the determinants between the interactions of Rab GTPases and Yip1p family members and Yop1p, I discovered an exciting finding. Rabs stably associate with membranes by virtue of their post-translational modification; the addition of two C20 lipid moieties (geranylgeranyl groups) onto conserved C-terminal cysteines of the protein. The prenylation of Rab proteins is catalyzed by Geranylgeranyltransferase type II (GGTase II) as illustrated in Figure 3 [10].



**Figure 4. Rab mutants used in this study.** This figure shows the mutations and predicted modifications of Rab proteins that we have examined in these studies. The two terminal cysteines of Rabs were substituted for CAAX boxes with either a CTIM sequence (farnesyl modification) or a CIIL (single geranylgeranyl modification) sequence. In addition, we mutated one of the terminal cysteines to a serine which should result in the single prenylation of the Rab. Furthermore, the two cysteines were deleted rendering the Rab protein unprenylated.

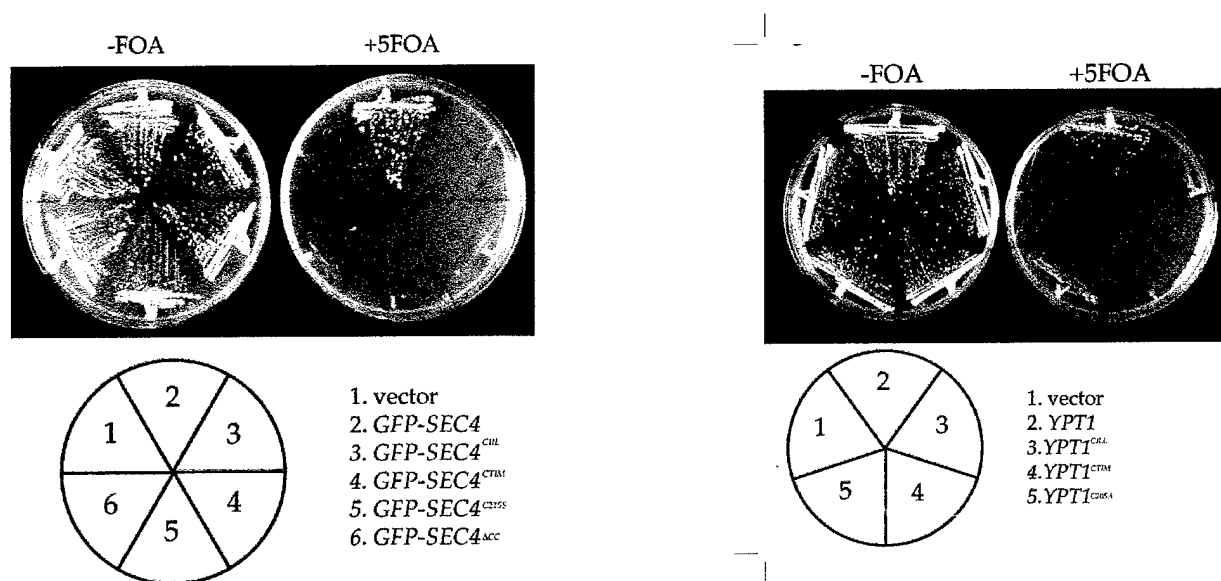
Since my previous data suggested that Yip1p family members and Yop1p did not interact with unprenylated Rabs I decided to investigate the effect of different lipid modifications on Rab and the interactions with Yip1p. I cloned several different Rab proteins with different C-termini (see Figure 4).

Other small G proteins such as Ras and Rho family of proteins contain a CAAX box at their C-terminus (C-cys, A-aliphatic amino acid, X= A, C, E, M, L, S, or V) [11]. Proteins ending in -CAAX where X=A, C, E, M, S or V (such as H-Ras, K-Ras and yeast Ras1p and Ras2p) are farnesylated by the enzyme Farnesyltransferase (FTaseI). Proteins ending in -CAAL (which includes Rho2p Rho1p, cdc42) are geranylgeranylated by Geranylgeranyl transferase I (GGTaseI). FTaseI and GGTase I transfer either farnesyl diphosphate (FPP 15 carbon lipid) or geranylgeranyl diphosphate (GGPP 20 carbon lipid) to the cysteine residue of the CAAX box (respectively). By substituting the terminal cysteines with CAAX boxes, or by mutating one of the cysteines to a serine, I was able to study singly prenylated Rabs and their interaction with Yip1p. Yip1p specifically interacts with double prenylated Sec4p (yeast homolog of Rab3A) and do not interact with singly prenylated Sec4p (See Table 2).

BAIT	PREY	RESULT
SEC4	YIP1	+++
SEC4 <sup>CTIM</sup>	YIP1	-
SEC4 <sup>CHL</sup>	YIP1	-
SEC4 <sup>ACC</sup>	YIP1	-

**Table 2. Two hybrid interactions of Yip1p with Sec4p and Sec4p mutants.**  $\beta$ -galactosidase activity was determined by filter assay. Pairs of constructs were coexpressed in the reporter strain Y190. Plus represents a positive activity rated according to the following criteria (+++) activity detected after 30 min, (++) activity detected after 90 min, and (+) activity detected after 5 hours, and minus (-) a negative indication of activity. At least 30 independent transformants were tested for each pair.

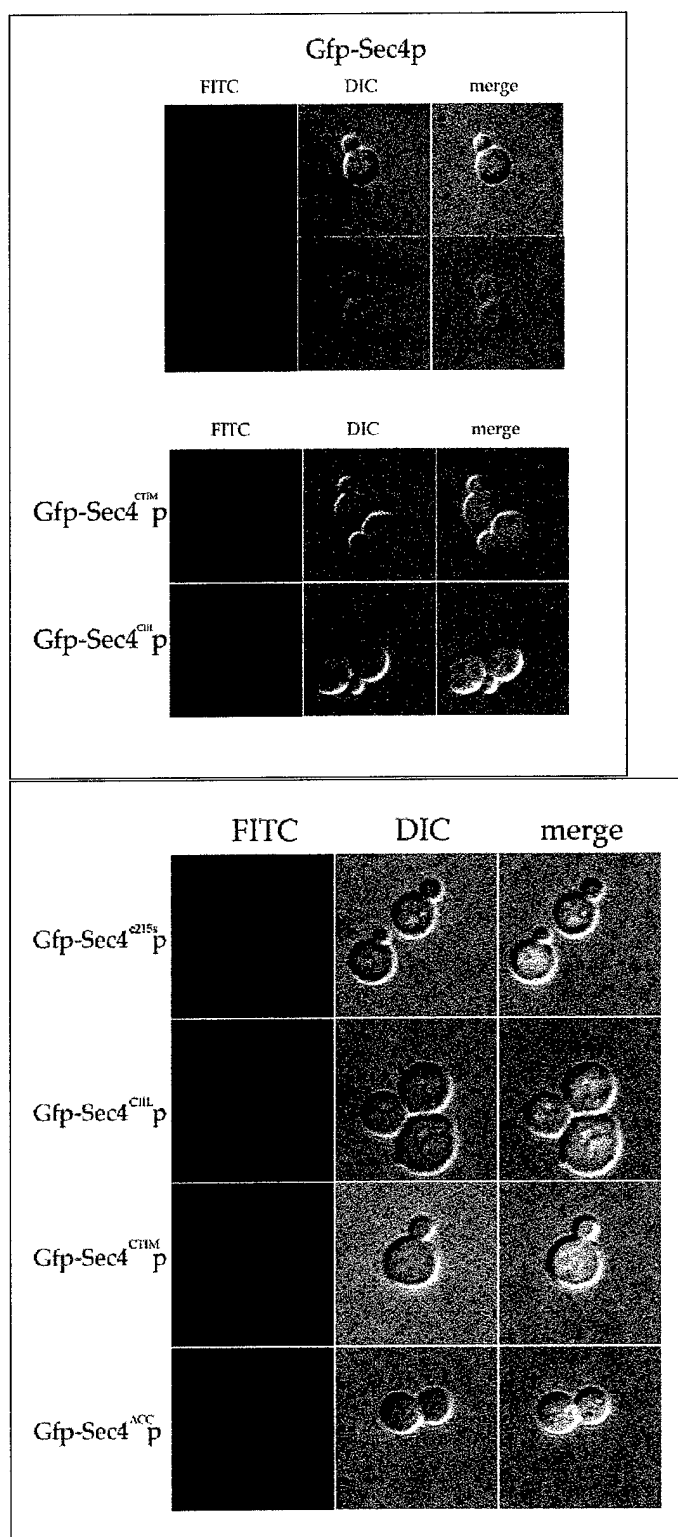
In order to study the functionality of these mutants, I decided to focus on the Rab3A homolog in yeast Sec4p, and Ypt1p, the homolog of Rab1. These two genes are essential in yeast. I transformed the various Rab mutants into disruption strain of *SEC4* and *YPT1* that have a carrier plasmid (WT *SEC4* or WT *YPT1* in *URA3* plasmids). By plasmid shuffle, I exchanged the mutant plasmids for the WT plasmid. The results of these experiments are shown in Figure 5. None of the singly prenylated Rabs were able to function as the only source of either *SEC4* or *YPT1*. Double prenylation is a requirement for Rab function.



**Figure 5. Singly prenylated Sec4p and Ypt1p are not functional.** In order to test functionality, we transform the indicated constructs into the tester strain RCY1507 (*MATa ura3-52 leu2-3,112 his3Δ200 SEC4Δ::HIS3 [URA3 CEN SEC4]*) or RCY1510 (*MATa ura3-52 leu2-3,112 his3Δ200 YPT1Δ::HIS3 [URA3 CEN YPT1]*). Transformants were streaked into plates containing 5-FOA. Under this condition, only wild type *GFP-SEC4* and *YPT1* are able to grow indicating that singly prenylated Rabs are not able to function as the only copy in the cell.

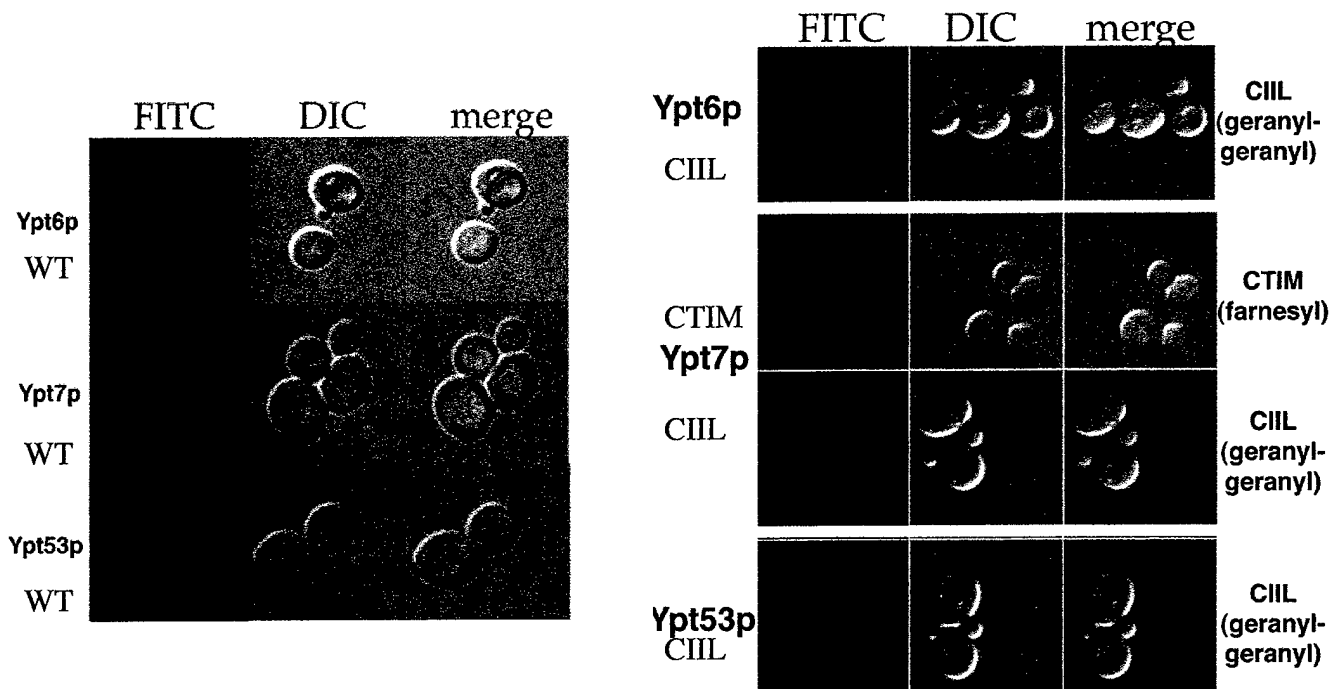
A hallmark of Rab GTPases is their specific association with subcellular compartment in the cell. I therefore investigated if various singly prenylated Rabs were correctly localized to their characteristic organelle membrane. I cloned the wild type and the singly prenylated mutants of a representative set of the

11 Rabs in yeast: Sec4p, Ypt1p and other yeast Rabs such as Ypt6p (Rab6), Ypt7p (Rab7), Ypt53p (Rab5 with a GFP tag at the N-terminus. These constructs were cloned into CEN vectors (centromeric plasmids that contain a centromere and are single copy) in order to study them at wild type levels. I transformed yeast with these constructs and examined their localization by fluorescent microscopy. These data are shown in Figures 6-9. As it is evident in these figures, each wild type Rab has a very specific localization in yeast. Sec4p is located in secretory vesicles at the bud tip. Ypt6p is localized at the Golgi reflected by the fluorescent punctated structures. Ypt7p is localized at the vacuole. The endocytic Rabs, Ypt53p is located at endosomes both early and late. However, none of the mutant Rabs localize correctly. The localization of the singly prenylated mutants was rather non-specific. In summary, these results suggest that double prenylation is a requirement for Rab protein localization and function. In addition, I have identified Yip1p as a specific factor that interacts with di-geranylgeranylated Rabs.



**Figure 6. Singly prenylated Sec4p does not localize to the WT compartment.** The localization of GFP-Sec4p and the GFP-Sec4p mutants was analyzed by fluorescent microscopy. As shown in Figure 4a, the fluorescent pattern of the GFP-Sec4pCAAX (both CTIM and CIIL sequences) constructs reveals reticular and punctated structures that are far from the classical WT localization of Sec4p. GFP-Sec4C215Sp revealed a rather non-specific signal that is similar to the unprenylated Sec4 (GFP-Sec4ACCp). In these experiments the cells were incubated with Hoescht stain for five minutes prior to the microscopy in order to visualize





**Figure 8: Singly prenylated Ypt6p, Ypt7p and Ypt53p do not localize to wild type compartments.** The localization of WT and mutant RabCAAX proteins was examined by fluorescent microscopy. Singly prenylated GFP-Ypt6p, GFP-Ypt7 and GFP-Ypt53p do not resemble the WT localization of the doubly prenylated proteins. For Ypt7p, the mutant reflected a very bright non-specific fluorescence. The same was true for Ypt53p and Ypt6p (data not shown for the CTIM sequences). Ypt53CIIL showed some endosomal staining, however the pattern differs significantly from the WT Ypt53p.

In the progress of my work, I have characterized Yip1p and Yop1p as Rab interacting factors which are conserved in evolution. However, the role of these proteins remains unknown. The ultimate goal of my research proposal is to elucidate the role of membrane traffic in malignant transformation and cancers. *S. cerevisiae* has served me as an excellent model organism to dissect the molecular analysis of Rab proteins with Yip1p and Yop1p. I have started a mutagenesis approach on Yip1p and Yop1p. In Figure 9, I show the results of random mutagenesis of Yip1p soluble domain. I am currently looking for phenotypes associated with these mutants. In the meantime since I have found that Yip1p interacts specifically with doubly prenylated Rabs I will start mutating the membrane domain to hopefully identify the Rab binding domain on the protein. The idea is to generate tools in order to carry out genetic studies on the physiological role of these proteins. Furthermore, I have cloned several mammalian Rabs: Rab3A, Rab8, Rab13 and Rab4 and TB2 (human homolog of Yop1p). In the next year, I will incorporate TB2 and TB2 mutants and Yip1A into transfection assays and assess their role in MCF7 cells.



### **KEY RESEARCH ACCOMPLISHMENTS:**

- Characterization of the Yip3p, yeast homolog of PRA1, a protein identified to associate with Rab3A
- Identification and characterization of a Yip1p-like family.
- Cloning of mammalian Yip1A (homolog of Yip1p). We have shown that that mammalian Yip1A associates with mammalian Rabs.
- We have localize Yip1A in mammalian cells
- Identification of the di-geranylgeranylation of Rab proteins as a requirement for Rab protein localization and function.
- I have found that Yip1p specifically interacts with doubly prenylated Rabs.
- Cloning of mammalian Rab3A, Rab8, Rab13, Rab4, Rab5, Rab9 and mammalian homolog of Yop1p (TB2).

### **REPORTABLE OUTCOMES:**

Outcomes that have resulted from this research :

1. Journal article publication : Calero,M., Whittaker,G.R and Collins,R.N..(2001) "Yop1p, the yeast homolg of the polyposis locus protein, interacts with Yip1p and negatively regulates cell growth". *JBC* **2276**(15): 12100-12.
2. Journal article publication : Calero,M. and Collins,R.N..(2002) "Saccharomyces cerevisiae Pra1p/Yip3p Interacts with Yip1p and Rab Proteins". *Biochem Biophys Res Commun* **290**(2): 676-81.
3. Journal article publication : Calero,M., Winand, N.J. and Collins,R.N..(2002) "Identification of the novel proteins Yip4p and Yip5p as Rab GTPase interacting factors". *FEBS Lett.* **515**(1-3):89-98.
4. Journal article in preparation: Calero,M., Collins,R.N.. "Double prenylation is a requirement for Rab protein localization and function"
4. Poster presented at the annual meeting of the field of Pharmacology, Cornell University: "Double prenylation is a requirement for Rab protein localization and function"

## **CONCLUSIONS:**

There is a growing appreciation that many proteins involved in intracellular protein trafficking are linked to uncontrolled cellular proliferation by diverse mechanisms [12-14]. The ultimate goal of my research is to elucidate the role of TB2, Yip1A and Rab proteins in these events. In this past year, I have further characterize the association of Yip1p and related proteins with Rab GTPases. More significantly, I have shown an *in vivo* result suggesting Yip1p as a negative regulator of Sec4p, the yeast homolog of Rab3A. Rab3A has been shown to be accumulated in MCF7 cells. Moreover, I have shown that Yip1p is a factor that interacts with the di-geranylgeranylated Rabs and not with the mono-prenylated Rabs. This results is significant and has implications in cancer treatmen. The suggestion is that prenyl lipid moieties provide much more than merely membrane anchors, but rather they are a critical part for the function, the localization and the interaction with Yip1p of Rab GTPases. This finding may be extrapolated to other G-proteins involved in cellular transformations such as Ras. The identification and characterization membrane proteins, such as Yip1p and Yop1p, that interact with GTPases will give us a further understanding in the ways of the localization and functionality of these signaling molecules. . The next and final year of my funding awaits exciting experiments. The studies will be aimed to clarify roles and control mechanisms of TB2, Yip1A in membrane transport and their role in cancer.

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## **APPENDICES**

## *Saccharomyces cerevisiae* Pra1p/Yip3p Interacts with Yip1p and Rab Proteins

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Received December 5, 2001

**The regulation of membrane traffic involves the Rab family of Ras-related GTPases, of which there are a total of 11 members in the yeast *Saccharomyces cerevisiae*. Previous work has identified PRA1 as a dual prenylated Rab GTPase and VAMP2 interacting protein [Martinic *et al.* (1999) *J. Biol. Chem.* 272, 26991–26998]. In this study we demonstrate that the yeast counterpart of PRA1 interacts with Rab proteins and with Yip1p, a membrane protein of unknown function that has been reported to interact specifically with the Rab proteins Ypt1p and Ypt31p. Yeast Pra1p/Yip3p is a factor capable of biochemical interaction with a panel of different Rab proteins and does not show *in vitro* specificity for any particular Rab. The interactions between Pra1p/Yip3p and Rab proteins are dependent on the presence of the Rab protein C-terminal cysteines and require C-terminal prenylation. © 2002 Elsevier Science**

**Key Words:** PRA1; YIP3; GDI; Rab; membrane traffic; yeast; YIP1.

Rab GTPases form the largest branch of small GTPases in the Ras superfamily and are found in all eukaryotic organisms (1). Rab proteins perform essential functions in different membrane transport pathways of the cell such as vesicle biogenesis (2), targeting and fusion of membrane-bound containers (3), and the association of organelles with motor proteins (4).

As with other members of the Ras superfamily, the intrinsic interconversion rates between the GDP- and GTP-bound forms of the molecule are slow, and are regulated by accessory factors such as Guanine nucleotide Exchange Factors (GEFs) and GTPase Activating Proteins (GAPs). In addition to their cycle of nucleotide binding and hydrolysis, Rab proteins also undergo cycles of membrane association and dissociation. Rab proteins stably attach to membranes by virtue of their post-translational prenylation modification; the attachment of two C20 geranylgeranyl groups onto C-terminal

amino acid cysteines of the protein. The Rab protein can be removed from the membrane through the action of Rab-GDI (GDI). GDI is a soluble protein whose recognition site consists of both the GDP-bound Rab and its prenylation moiety (5). The heterodimer of GDP/Rab-GDI enables the Rab protein to be retrieved through the cytosol back onto membranes. The membrane recruitment reaction of Rabs is highly specific, each organelle of the secretory and endocytic pathways is found to associate with a particular Rab protein(s).

To date, many of the Rab accessory factors that have been identified are soluble proteins whose activity can be assigned to defined classes such as effectors, GEFs, GAPs etc. based on their effect on the GDP-GTP interconversion rates. Recently, several Rab-interacting membrane proteins have been identified. These include, Yip1p, PRA1, rab5ip, and Yop1p (6–9). The existence of such proteins raises a question as to their effect on the Rab GTPase cycle of nucleotide binding and localization. There are at least two intervention points in the Rab cycle which may require membrane proteins. The first of these is the dissociation of the cytosolic Rab-GDI heterodimer and subsequent recruitment of the free Rab protein onto membranes. This reaction is specific and is accompanied by the release of GDI, hence the factor that mediates this event has been termed GDI displacement factor (GDF) (10, 11). The second intervention point may be a membrane recycling factor which aids in Rab membrane dissociation. Although GDI is capable of removing Rabs from membranes *in vitro*, this process may be aided *in vivo* by a membrane-associated recycling factor (12).

Rat PRA1 was isolated previously as a Rab3/Rab1 interacting protein (7) however the ability of its yeast homolog Pra1p/Yip3p to physically interact with Rab proteins has not been tested to date. We have tested Pra1p/Yip3p for specificity of the interaction between Yip1p and Rabs. We find that mutations preventing C-terminal prenylation can prevent association of Rabs and Pra1p/Yip3p. Furthermore, we find that the binding of Rab proteins to Pra1p/Yip3p is nonspecific; *in*

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TABLE 1  
*S. cerevisiae* Strains Used in This Study

Strain	Genotype	Source
RCY427	<i>MATa ura3-52 leu2-3, 112::LEU2 P<sub>GAL1/10</sub>GST</i>	This study
RCY442	<i>MATa ura3-52 leu2-3, 112::LEU2 P<sub>GAL1/10</sub>GST-YPT7</i>	This study
RCY539	<i>MATa ura3-52 leu2-3, 112::LEU2 P<sub>GAL1/10</sub>GST-YIP1</i>	This study
RCY693	<i>MATa ura3-52 leu2-3, 112::LEU2 P<sub>GAL1/10</sub>GST-YPT10</i>	This study
RCY694	<i>MATa ura3-52 leu2-3, 112::LEU2 P<sub>GAL1/10</sub>GST-YPT11</i>	This study
RCY695	<i>MATa ura3-52 leu2-3, 112::LEU2 P<sub>GAL1/10</sub>GST-YPT31</i>	This study
RCY696	<i>MATa ura3-52 leu2-3, 112::LEU2 P<sub>GAL1/10</sub>GST-YPT32</i>	This study
RCY697	<i>MATa ura3-52 leu2-3, 112::LEU2 P<sub>GAL1/10</sub>GST-YPT52</i>	This study
RCY698	<i>MATa ura3-52 leu2-3, 112::LEU2 P<sub>GAL1/10</sub>GST-YPT6</i>	This study
RCY699	<i>MATa ura3-52 leu2-3, 112::LEU2 P<sub>GAL1/10</sub>GST-SEC4</i>	This study
RCY700	<i>MATa ura3-52 leu2-3, 112::LEU2 P<sub>GAL1/10</sub>GST-YPT1ΔC</i>	This study
RCY701	<i>MATa ura3-52 leu2-3, 112::LEU2 P<sub>GAL1/10</sub>GST-YPT1</i>	This study
RCY749	<i>MATa ura3-52 leu2-3, 112::LEU2 P<sub>GAL1/10</sub>GST-YPT7 [MBP-Pra1p/Yip3p pRS426 pRC1050]</i>	This study
RCY849	<i>MATa ura3-52 leu2-3, 112::LEU2 P<sub>GAL1/10</sub>GST-YIP1 [MBP-Pra1p/Yip3p pRS426 pRC1050]</i>	This study
Y190	<i>MATa gal4Δ gal80Δ trp1-901 ade2-101 ura3-52 leu2-3, 112 URA3::GAL10 → LacZ, LYS2::GAL10 → HIS3 cyh<sup>R</sup></i>	Elledge laboratory

*vitro*, Pra1p/Yip3p will associate with a variety of Rabs. In addition to Rab proteins, Pra1p/Yip3p also associates with Yip1p and we demonstrate this interaction in cellular lysates.

## MATERIALS AND METHODS

**Yeast strains and media.** The *S. cerevisiae* strains used in these studies are listed in Table 1.

**Two-hybrid assay.** The ORF sequences were subcloned into pAS1-CYH2 or pAS2-1 and pACTII for "bait" and "prey" constructs respectively. The yeast strain Y190 was used to assay for interacting clones (13). pAS1-CYH2 constructs pRC38, pRC22, pRC27, pRC34, pRC33, pRC804, pRC805, pRC9.8, pRC29, pRC31, pRC25, pRC787, pRC762 express Ypt7p, Yif1p, Ypt11p, Ypt52p, Ypt53p, Ypt1p, Ypt51p, Sec4p, Ypt31p, Ypt32p, Ypt10p, canine Rab1A, and human Rab5, respectively. pACTII constructs pRC40, clone 11.1 express Yip3p exon 2, and Yip1p, respectively.

**Coprecipitation experiments.** Rab proteins as indicated were expressed as GST-fusion proteins under the control of the *GAL*<sub>1/10</sub> promoter in yeast. These strains coexpressed a plasmid containing MBP-tagged Yip3p. The experimental protocol was as described in (9). Strains used for pulldown experiments were grown overnight in 50 ml of selective medium containing galactose as carbon source (SGal) to an absorbance of ~0.7 A<sub>600</sub>. Cells were harvested by centrifugation at 4°C and washed in 1 ml of ice-cold buffer (10 mM Tris, pH 7.5, 10 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>). For all pulldowns, cell pellets were resuspended in 100 μl of ice cold lysis buffer (20 mM KPi, pH 7.5, 80 mM KCl, 1 mM EGTA, 2% glycerol, 0.8% Tween 20) containing protease inhibitors (10 mM PMSF, 10 μg/ml pepstatin A) before lysis with glass beads. A total detergent solubilized extract was generated by incubating lysates with an additional 1 ml of lysis buffer for 10 min at 4°C. Detergent-solubilized lysates were cleared by two sequential centrifugation steps in a microfuge for 5 min at 13,000 rpm. Samples were incubated with rocking for 30 min at 4°C with 20 μl of amylose resin (New England Biolabs). The bead-bound material was washed with four times with lysis buffer. Similar procedures were followed for GST-pulldowns except glutathione *S*-Sepharose resin (Pharmacia) was used to isolate the GST-tagged proteins. Proteins were eluted from the beads by boiling in SDS sample buffer. The proteins were analyzed by 10% SDS-PAGE gel electrophoresis and Western blotting with anti-GST antibody to detect the presence of

the GST-tagged Rab proteins (for these purposes the anti-GFP antibody Santa Cruz Cat. No. SC-8334, lot G030 was used, this antibody recognized GST in Western blots with far higher avidity than GFP). Anti-MBP antibody (gift of G. R. Whittaker) was used at 1:6000 to detect MBP-tagged proteins. Secondary alkaline phosphatase-conjugated goat anti-rabbit antibodies (Kirkegaard and Perry Laboratories) were added in blocking buffer, followed by washing and chromogenic blot development with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (both from Bio-Rad) substrates in AP buffer (100 mM Tris, pH 9.5, 100 mM NaCl, and 5 mM MgCl<sub>2</sub>).

Rab protein expression under the control of the *GAL*<sub>1/10</sub> promoter was achieved by subcloning the ORF containing the Rab protein in frame with GST into the vector pRC337. GST-Ypt10p, -Ypt11p, -Ypt31p, -Ypt32p, -Ypt52p, -Ypt6p, -Sec4p, -Ypt1ΔC, -Ypt1p, -Yip1p were expressed from the constructs pRC696, pRC697, pRC698, pRC699, pRC700, pRC701, pRC702, pRC711, pRC1016, and pRC726, respectively. These constructs were linearized with a restriction enzyme and integrated into the genome at the *LEU2* locus. Expression of a GST fusion protein of the correct *M<sub>r</sub>* was determined by growing the cells in media containing 2% galactose as a carbon source. The plasmid containing MBP tagged Yip3p (pRC1050) was constructed by overlap PCR to insert a MBP tag cassette immediately after the initiating methionine in order to express the fusion protein under the control of an endogenous promoter and terminator in the yeast vector pRS426. The PRA1/YIP3 template used for the PCR was an intronless version of the gene created with the primers RNC77 (5'-TTCTATTACCAGAGTACT-TGGTATCGAATTGTTTCATTTGAG-3') and RNC78 (5'-CGATAC-CAAGTACTCTGGTAATAGAATTTTACAGC-3') in order to precisely eliminate the intron with no change in coding sequence.

## RESULTS AND DISCUSSION

### *Pra1p/Yip3p Interacts with Multiple Rab Proteins*

Martincic *et al.* (7) have previously identified Rat PRA1 as a factor that interacts specifically with Rab3 and Rab1. We wished to extend these observations to the yeast counterpart of PRA1. We performed a deliberate pairwise testing of constructs; including every known Rab ORF (11 total) in *S. cerevisiae*. Interactions

TABLE 2  
Pattern of Two-Hybrid Interactions of Rabs  
with YIP3/PRA1

Bait	Prey		
	YIP3	YIP3 exon 2	GDI1
YPT6	+++	+++	++
YPT7	—	—	+
YPT11	+++	+++	+/-
YPT52	+++	+++	+++
YPT53	+++	+++	+++
YPT1	+++	+++	+++
YPT51	+++	+++	+++
SEC4	+++	+++	+++
SEC4ΔC	—	—	—
YPT31	+++	+++	+++
YPT32	+++	+++	+++
YPT10	+++	+++	+++
Rab1	+++	+++	+++
Rab5	+++	+++	+++

Note.  $\beta$ -Galactoside activity was determined by filter assay. Pairs were coexpressed in the receptor strain Y190. Plus represents a positive activity rated according to the following criteria (+++) activity detected after 30 min, (++) activity detected after 90 min, and (+) activity detected after 5 h, and minus (—) is a negative indication of activity. At least 30 independent transformants were tested for each pair.

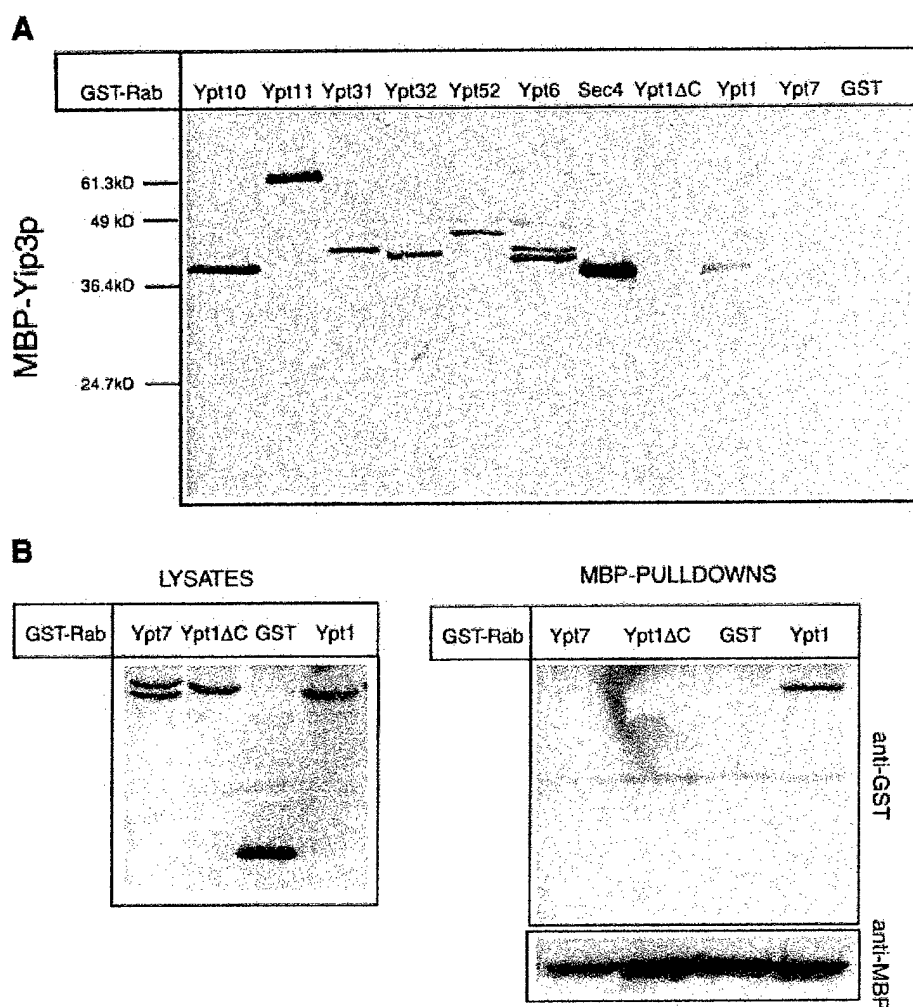
with Pra1p/Yip3p were observed for all yeast Rabs (Table 2) except for YPT7 and also the mammalian Rabs, Rab1 and Rab5. These data show that Pra1p/Yip3p interacts with multiple Rab proteins from different species.

The Y2H results were confirmed with an independent method of detecting protein-protein interactions. For these experiments (Fig. 1A) a representative selection of GST-tagged Rab proteins were expressed in cells containing MBP-tagged Yip3p. Untagged GST was expressed as a negative control. The cellular lysates were incubated with amylose resin for 30 min at 0°C to pull-down the MBP-Yip1p protein. After extensive washing, the bead-bound material was analyzed by SDS-PAGE and Western blotting. The Western blots were probed with anti-GST polyclonal antibody to detect any associated Rab proteins. Association of Pra1p/Yip3p was detected with GST-Sec4p, GST-Ypt1p, GST-Ypt6p, GST-Ypt10p, GST-Ypt31p, GST-Ypt32p, and GST-Ypt52p but not to GST alone, GST-Ypt1ΔC or GST-Ypt7p. These results parallel the data obtained in the two-hybrid assay and show that Pra1p/Yip3p in cellular lysates binds to diverse Rab proteins in a manner dependent upon C-terminal prenylation. Ypt7p was the only Rab protein not to interact with Pra1p/Yip3p in cellular lysates. To eliminate the possibility this was due to expression levels of the GST-Ypt7p construct we analyzed the expression levels of this construct in cellular lysates. These data are shown in Fig. 1B, which shows that GST-Ypt7p, GST-

Ypt1ΔCp, GST-Ypt1p, and GST alone were expressed at comparable levels in the respective cellular lysates. An equivalent amount of MBP-Yip3p was precipitated in these experiments as revealed by an anti-MBP antibody probe of the membranes. We conclude that Ypt7p is unique amongst Rab proteins in its inability to bind to Pra1p/Yip3p in cellular lysates. Perhaps Ypt7p does not possess the Pra1p/Yip3p binding motif shared by all other Rab proteins. Other possible explanations are that the Pra1p/Yip3p binding site on Ypt7p is masked by association with a Ypt7p-specific factor or that interactions cannot be detected with the tagged constructs used in our system. Purification of Pra1p/Yip3p and demonstration of the direct nature of its interaction with Rab proteins is required for further clarification of this question.

YIP3/PRA1 is unusual amongst yeast genes in that the gene organization consists of two exons, potentially reflecting domain organization of the protein. We used the TMPred program ([http://www.ch.embnet.org/software/TMPRED\\_form.html](http://www.ch.embnet.org/software/TMPRED_form.html)) to make a prediction of membrane-spanning regions and their orientation for YIP3. The TMPred algorithm is based on the statistical analysis of TMbase, a database of naturally occurring transmembrane proteins using a combination of several weight-matrices for scoring (14). The results of this analysis are shown in Fig. 2. Pra1p/Yip3p is a small 19.4-kDa protein with significant hydrophobic segments that potentially span or are inserted into the membrane. The predicted topology for Pra1p/Yip3p suggests that it exists with a significant soluble N-terminal domain that is oriented toward the cytosol and a C-terminal domain where the hydrophobic segments are located. The topology for yeast Pra1p/Yip3p is very similar to that of mouse PRA1 which has recently reported to be a polytopic membrane protein with four transmembrane segments and a cytosolic N-terminus. Surprisingly, rat PRA1 has been reported to be present in both high speed supernatant and pellet fractions (15). It is difficult to imagine how a polytopic membrane protein can be present in a cytosolic fraction devoid of membranes. Perhaps rat PRA1 exists as a multimeric soluble complex where protein acyl motifs replace the environment of the lipid bilayer enabling the to exist in a cytosolic state? We tested the domain represented by exon 2 in isolation for Rab protein interaction. The exon 2 domain replicated the Y2H interactions observed for full length YIP3/PRA1, suggesting that Rab proteins bind to the C-terminal hydrophobic domain and exclude a role for the N-terminus represented by exon 1 (Table 2).

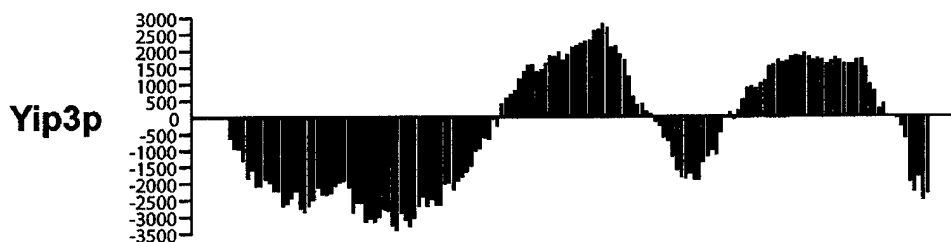
Our data demonstrate that Pra1p/Yip3p interacts with multiple different Rab proteins. This data, together the fact that the human homolog PRA1 interacts with the v-SNARE protein synaptobrevin, builds up a picture of Pra1p/Yip3p playing a role in membrane traffic events. In addition to Rab proteins, Pra1p/



**FIG. 1.** Biochemical analysis of Pra1p/Yip3p interactions with Rabs: MBP-Yip3p interacts with fully post-translationally modified Rab proteins. (A) Lysates were prepared from cells expressing various GST-Rab constructs as indicated. Detergent-solubilized lysates were incubated with amylose resin for 30 min at 4°C as described under Materials and Methods to pull-down the MBP-Yip3p. After four washes, the bead-bound material was subject to SDS-PAGE electrophoresis and analyzed by Western blotting. Membranes were probed with polyclonal anti-GST (1:800) to detect GST-Rab proteins. Relevant protein marker sizes are indicated. (B) MBP-Yip3p fusion protein was purified from lysates prepared from cells expressing GST-Ypt7p, GST-Ypt1ΔCp, GST alone, or GST-Ypt1p. Both total cell lysates and the MBP-pulldowns were Western blotted with anti-GST antibodies to detect the relative abundance of the GST fusion proteins. The MBP-pulldowns were additionally subject to Western blotting with anti-MBP antibodies to confirm the precipitation of MBP-Yip3p on the amylose resin. Only GST-Ypt1p, but not GST-Ypt7p, GST-Ypt1ΔCp, or GST were observed to coprecipitate with MBP-Yip3p although these constructs were expressed at similar levels in the cellular lysates.

Yip3p has also been shown to interact with Rho and Ras small GTPases in a manner dependent on C-terminal prenylation (16). The *in vivo* significance of this data is unclear. Although conserved in evolution and ubiquitously expressed (17), *PRA1/YIP3* is not an essential gene in yeast perhaps indicating its function is in a supporting or mediator role. By binding prenylation groups on small GTPases, or other prenylated molecules, Pra1p/Yip3p has been suggested to act as a carrier protein mediating the intracellular movement of prenylated proteins (16), a function it could carry out alone or in concert with other binding partner(s). One possible hypothesis for Yip1p function is suggested by

the features of Pra1p/Yip3p interaction with Rabs demonstrated in this study, namely that interactions are (i) nonspecific and (ii) require the C-terminal cysteines which are the recipient sites for double geranylgeranylation. These features exactly mirror the requirements for Rab interaction with Rab-GDI and suggest that Pra1p/Yip3p can directly compete with Rab-GDI for Rab protein interactions on the membrane. Such an outcome has been suggested by the study of Hutt *et al.* (15) although the physiological significance of this data is unclear. In addition, the finding that Pra1p/Yip3p can bind prenylated Rho small GTPases suggest that Pra1p/Yip3p could simi-



**FIG. 2.** TMpred plot of Pra1p/Yip3p. The TMpred plot of Pra1p/Yip3p was generated using the program TMpred with a 17-residue minimal and 33 residue maximal length of the hydrophobic part of the transmembrane helix and shows the relative location of the y-axis the relative hydrophobicity (positive values) or hydrophilicity (negative values). Sequence data indicate a predicted topology for Pra1p/Yip3p with a significant soluble N-terminal domain that is oriented toward the cytosol and a C-terminal domain where the hydrophobic segments are located.

larly directly compete with Rho-GDI for Rab protein interactions on the membrane.

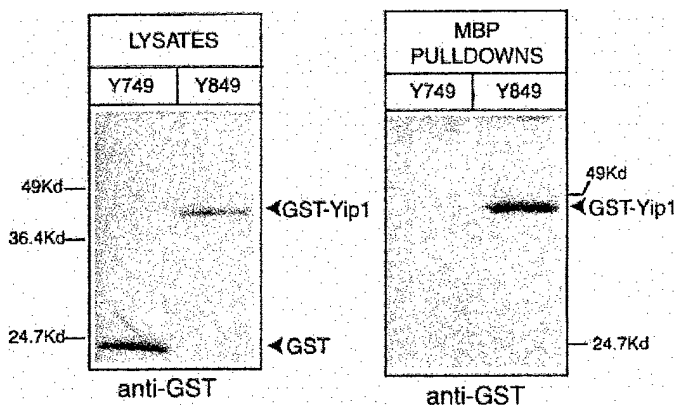
Our biochemical data reveal that the yeast PRA1-related protein is capable of binding to a common determinant shared by multiple Rab proteins with the exception of Ypt7p. These data are in agreement with the finding that rat PRA1 interacts specifically with Rab3A and Rab1. Rat PRA1 does not interact with Rho or Rac although is able to bind Rab proteins where the usual di-cysteine motif has been replaced with the CAAX motif for mono-geranylgeranylation (7). This is in contrast to the finding that yeast Pra1p/Yip3p interacts with Rho proteins (16), although this study did not examine or compare the interaction with Rab proteins. Our results demonstrate these Pra1p/Yip3p interactions are conserved across evolution, not only does YIP3 interact with yeast Rab proteins, it will similarly interact with a mammalian Rab protein and similar interactions by Y2H have been reported for a human homolog of PRA1 (18). Demonstration of the direct or indirect nature of the PRA1 interactions is required for further resolution of these questions since all experiments carried out to date have been performed in cell extracts.

#### *Pra1p/Yip3p Interacts with Yip1p*

In addition to Rab proteins, Pra1p/Yip3p has also been observed to interact with Yip1p by Y2H (19–21). We decided to test this interaction biochemically in co-precipitation experiments. For these experiments, Yip1p was tagged with GST and Yip3p was tagged with MBP. GST alone was used as a control. An amylose resin pull-down from detergent solubilized lysates of cells expressing either MBP–Yip3p together with GST alone or GST-tagged Yip1p revealed that Yip1p could be specifically co-precipitated (Fig. 3). Our data therefore confirm and extend the Y2H observations identified in high throughput screens for Pra1p/Yip3p.

In addition to pleiotropic Rab interactions *in vitro*, our analysis suggests that YIP3 is able to interact with the essential membrane protein Yip1p. Human PRA1

can also interact with Epstein–Barr virus BHRF1, a homologue of Bcl-2 (22) and Piccolo, a novel component of the presynaptic cytoskeletal matrix (23). Yip1p has also been observed by biochemical experiments and Y2H to interact with both Yif1p and Yop1p (9, 24); and by Y2H with YIP3, YGL198W, YGL161C, YPL095C, GCS1, and YLR324W (19–21). The relevance of these demonstrated and potential interactions is obscure, although Yip1p, Yif1p and Pra1p/Yip3p have been observed to be selectively packaged into COPII vesicles *in vitro* (25), perhaps providing a link between YIP1 family members, Rab proteins and the vesicle docking and fusion machinery. Further work will be required to clarify the complex and confusing issues surrounding these conserved proteins and to understand the physiological role of PRA1/YIP3 and its mechanism of action.



**FIG. 3.** Biochemical analysis of Pra1p/Yip3p interactions with Yip1p. Lysates were prepared from cells expressing either GST alone or GST–Yip1p together with MBP–Yip3p. Detergent solubilized lysates were incubated with amylose resin for 30 min at 4°C as described under Materials and Methods. After washing, the bead-bound material was subject to SDS–PAGE and analyzed by Western blotting. Membranes were probed with polyclonal anti-GST (1:800) to detect GST–Yip1p. Relevant protein marker sizes are indicated. GST–Yip1p was detected in RCY849 but not on RCY749 after MBP-pull-downs.

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# Identification of the novel proteins Yip4p and Yip5p as Rab GTPase interacting factors

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**Abstract** The Rab GTPases are key regulators of membrane traffic. Yip1p is a membrane protein of unknown function that has been reported to interact with the Rabs Ypt1p and Ypt31p. In this study we identify Yif1p, and two unknown open reading frames, Ygl198p and Ygl161p, which we term Yip4p and Yip5p, as Yip1p-related sequences. We demonstrate that the Yip1p-related proteins possess several features: (i) they have a common overall domain topology, (ii) they are capable of biochemical interaction with a variety of Rab proteins in a manner dependent on C-terminal prenylation, and (iii) they share an ability to physically associate with other members of the YIP1 family. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Rab; YIP1; YIP4; YIP5; YGL198W; YGL161C

## 1. Introduction

Rab GTPases form the largest branch of small GTPases in the Ras superfamily and are found in all eukaryotic organisms [1]. Rab proteins perform essential functions in different membrane transport pathways of the cell such as vesicle biogenesis [2], targeting and fusion of membrane-bound containers [3], and the association of organelles with motor proteins [4].

Like other members of the Ras superfamily, the intrinsic interconversion rates between the GDP- and GTP-bound forms of the protein are regulated by accessory factors such as guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). In addition to their cycle of nucleotide binding and hydrolysis, Rab proteins also undergo cycles of membrane association and dissociation. Rab proteins stably attach to membranes by virtue of their post-translational prenylation modification: the attachment of two C20 geranylgeranyl groups onto C-terminal cysteines of the protein [5]. The Rab protein can be removed from the membrane through the action of Rab-GDP dissociation inhibitor (GDI). GDI is a soluble protein whose recognition site consists of both the GDP-bound Rab and its prenylation moiety [6]. The heterodimer of GDP/Rab-GDI enables the Rab protein

to be recycled through the cytosol back onto membranes for subsequent rounds of transport. The membrane recruitment reaction of Rabs is highly specific, each organelle of the secretory and endocytic pathways is found to associate with a particular Rab protein(s).

To date, many of the Rab interacting proteins that have been identified are soluble factors whose activity can be assigned to defined classes such as effectors, GEFs, GAPs etc. based on their ability to modulate the Rab GTPase cycle. Recently, several Rab interacting membrane proteins have been identified. These include Yip1p, PRA1, rab5ip and Yop1p [7–10]. The existence of these proteins raises the exciting possibility that they are involved in regulating Rab function on membranes or perhaps modulate the association of Rab proteins with membranes. In this study, we have focused on one of this class of membrane proteins, Yip1p. Using Yip1p as a departure point we have identified YIP1-related sequences and demonstrate that the proteins encoded by these sequences have common characteristics and constitute a protein family. Because Yip1p is the founder member or prototype for this family we have termed it the YIP1 family. For small membrane proteins such as Yip1p, identification of homologs cannot be confidently predicted based on primary sequence comparison alone. This is due to the fact that large stretches of the protein consist of hydrophobic residues, reducing the complexity necessary for successful database mining. Our results define three additional criteria for a Yip1p-related protein. These criteria are a common domain topology, the ability to interact with Rab proteins in a manner dependent on C-terminal prenylation, and the ability to associate physically with other Yip1p family members. We demonstrate that Yif1p, and two unknown open reading frames (ORFs), YGL198W and YGL161C, share these features and qualify as YIP1 family members: we have termed these ORFs Yip4p and Yip5p respectively. The YIP1-related proteins are found across eukaryotes and YIP1 family members have both overlapping and distinct functions.

## 2. Materials and methods

### 2.1. Yeast strains and media

The *Saccharomyces cerevisiae* strains used in these studies are listed in Table 1. All yeast strains were manipulated as described in [11].

### 2.2. Yeast two-hybrid (Y2H) assay

The ORF sequences were subcloned into pAS1-CYH2 or pAS2-1 for 'bait' and pACT1 or pACT2 for 'prey' constructs respectively as listed in Table 2. pRC187 and pRC188 are two independent bait constructs which contain Yip1p. pRC1466 and pRC1467 are two in-

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**Abbreviations:** GDI, GDP dissociation inhibitor; GST, glutathione S-transferase; GAP, GTPase activating factor; GEF, guanine nucleotide exchange factor; Y2H, yeast two-hybrid; MBP, maltose binding protein; GFP, green fluorescent protein; 5-FOA, fluoroorotic acid

Table 1  
*S. cerevisiae* strains used in this study

Strain	Genotype	Source
RCY427	<i>MATa ura3-52 leu2-3,112::LEU2 P<sub>GAL1/10</sub>GST</i>	This laboratory [13]
RCY442	<i>MATa ura3-52 leu2-3,112::LEU2 P<sub>GAL1/10</sub>GST-YPT7</i>	This laboratory [13]
RCY539	<i>MATa ura3-52 leu2-3,112::LEU2 P<sub>GAL1/10</sub>GST-YIP1</i>	This laboratory [13]
RCY693	<i>MATa ura3-52 leu2-3,112::LEU2 P<sub>GAL1/10</sub>GST-YPT10</i>	This laboratory [13]
RCY694	<i>MATa ura3-52 leu2-3,112::LEU2 P<sub>GAL1/10</sub>GST-YPT11</i>	This laboratory [13]
RCY695	<i>MATa ura3-52 leu2-3,112::LEU2 P<sub>GAL1/10</sub>GST-YPT31</i>	This laboratory [13]
RCY696	<i>MATa ura3-52 leu2-3,112::LEU2 P<sub>GAL1/10</sub>GST-YPT32</i>	This laboratory [13]
RCY697	<i>MATa ura3-52 leu2-3,112::LEU2 P<sub>GAL1/10</sub>GST-YPT52</i>	This laboratory [13]
RCY698	<i>MATa ura3-52 leu2-3,112::LEU2 P<sub>GAL1/10</sub>GST-YPT6</i>	This laboratory [13]
RCY699	<i>MATa ura3-52 leu2-3,112::LEU2 P<sub>GAL1/10</sub>GST-SEC4</i>	This laboratory [13]
RCY700	<i>MATa ura3-52 leu2-3,112::LEU2 P<sub>GAL1/10</sub>GST-YPT1DC</i>	This laboratory [13]
RCY701	<i>MATa ura3-52 leu2-3,112::LEU2 P<sub>GAL1/10</sub>GST-YPT1</i>	This laboratory [13]
RCY765	<i>MATa ura3-52 leu2-3,112::LEU2 P<sub>GAL1/10</sub>GST [pRC1054]</i>	This study
RCY850	<i>MATa ura3-52 leu2-3,112::LEU2 P<sub>GAL1/10</sub>GST-YIP1 [pRC1053]</i>	This study
RCY780	<i>MATa ura3-52 leu2-3,112::LEU2 P<sub>GAL1/10</sub>GST [pRC1047]</i>	This study
RCY851	<i>MATa ura3-52 leu2-3,112::LEU2 P<sub>GAL1/10</sub>GST-YIP1 [pRC1047]</i>	This study
RCY873	<i>MATa ura3-52 leu2-3,112 [P<sub>GAL1/10</sub>GST-Yip4p CEN LEU2 pRC1578] [MBP-Yip4p pRS426 pRC1053]</i>	This study
RCY881	<i>MATa ura3-52 leu2-3,112 [P<sub>GAL1/10</sub>GST-Yif1p CEN LEU2 pRC1579] [MBP-Yip4p pRS426 pRC1053]</i>	This study
RCY1354	<i>MATa ura3-52 leu2-<math>\square</math> lys2-801 his3<math>\square</math>200 ade2-101 trp1-<math>\square</math>63 YIP1<math>\square</math>HIS [YCP50 YIP1 pRC1245]</i>	This study
Y190	<i>MATa gal4<math>\square</math> gal80<math>\square</math> trp1-901 ade2-101 ura3-52 leu2-3,112 URA3::GAL10<math>\square</math> LacZ, LYS2::GAL10<math>\square</math> HIS3 cyh<sup>R</sup></i>	Elledge laboratory

dependent prey constructs which contain Ygl161p (Yip5p). The yeast strain Y190 was used to assay for interacting constructs [12]. Due to batch variability in Y2H assays each complete experiment was carried out in a complete set which included positive and negative controls.

We also commonly observed variability in the Y2H system between two otherwise identical constructs and so two independently generated constructs were used to confirm interactions observed in our experiments. Pairs of plasmids were cotransformed into the yeast strain and

Table 2  
Plasmids used in this study

Name	Relevant features	Source
pRC38	pAS1-CYH2 Gal4-DNA binding domain Ypt7p fusion	This study
pRC22	pAS1-CYH2 Gal4-DNA binding domain Yif1p fusion	This study
pRC27	pAS1-CYH2 Gal4-DNA binding domain Ypt11p fusion	This study
pRC34	pAS1-CYH2 Gal4-DNA binding domain Ypt52p fusion	This study
pRC33	pAS1-CYH2 Gal4-DNA binding domain Ypt53p fusion	This study
pRC804	pAS1-CYH2 Gal4-DNA binding domain Ypt1p fusion	This study
pRC805	pAS1-CYH2 Gal4-DNA binding domain Ypt51p fusion	This study
pRC966	pAS1-CYH2 Gal4-DNA binding domain Sec4p fusion	Novick laboratory [27]
pRC29	pAS1-CYH2 Gal4-DNA binding domain Ypt31p fusion	This study
pRC31	pAS1-CYH2 Gal4-DNA binding domain Ypt32p fusion	This study
pRC25	pAS1-CYH2 Gal4-DNA binding domain Ypt10p fusion	This study
pRC1253	pAS1-CYH2 Gal4-DNA binding domain Dss4p fusion	Novick laboratory [27]
pRC225	pAS2-1 Gal4-DNA binding domain human Yip1p fusion	This study
pRC181	pAS1-CYH2 Gal4-DNA binding domain Yip4p fusion	This study
pRC977	pAS1-CYH2 Gal4-DNA binding domain Sec4 $\square$ Cp (Sec4p lacking C-terminal cysteines) fusion	Novick laboratory [27]
pRC187/pRC188	pAS1-CYH2 Gal4-DNA binding domain Yip1p fusion	This study
pRC957	pACTII Gal4-DNA activation domain Yip1p fusion	This study
pRC42	pACTII Gal4-DNA activation domain Yif1p fusion	This study
pRC44	pACTII Gal4-DNA activation domain Yip4p fusion	This study
pRC1464	pACTII Gal4-DNA activation domain Gdi1p fusion	Novick laboratory [27]
pRC1466/pRC1477	pACTII Gal4-DNA activation domain Yip5p fusion	This study
pRC1047	MBP tagged Yif1p <i>URA3</i> 2 $\square$ m (pRS426)	This study
pRC1049	MBP tagged Yip1p <i>URA3</i> 2 $\square$ m (pRS426)	This study
pRC1053/pRC1054	MBP tagged Yip4p <i>URA3</i> 2 $\square$ m (pRS426)	This study
pRC337	<i>LEU2</i> INT <i>GAL<sub>1/10</sub></i> GST (pRS305)	This laboratory [13]
pRC696	<i>LEU2</i> INT <i>GAL<sub>1/10</sub></i> GST-Ypt10p (pRS305)	This laboratory [13]
pRC697	<i>LEU2</i> INT <i>GAL<sub>1/10</sub></i> GST-Ypt11p (pRS305)	This laboratory [13]
pRC698	<i>LEU2</i> INT <i>GAL<sub>1/10</sub></i> GST-Ypt31p (pRS305)	This laboratory [13]
pRC699	<i>LEU2</i> INT <i>GAL<sub>1/10</sub></i> GST-Ypt32p (pRS305)	This laboratory [13]
pRC700	<i>LEU2</i> INT <i>GAL<sub>1/10</sub></i> GST-Ypt52p (pRS305)	This laboratory [13]
pRC701	<i>LEU2</i> INT <i>GAL<sub>1/10</sub></i> GST-Ypt6p (pRS305)	This laboratory [13]
pRC702	<i>LEU2</i> INT <i>GAL<sub>1/10</sub></i> GST-Sec4p (pRS305)	This laboratory [13]
pRC711	<i>LEU2</i> INT <i>GAL<sub>1/10</sub></i> GST-Ypt1DC (pRS305)	This laboratory [13]
pRC1016	<i>LEU2</i> INT <i>GAL<sub>1/10</sub></i> GST-Ypt1p (pRS305)	This laboratory [13]
pRC726	<i>LEU2</i> INT <i>GAL<sub>1/10</sub></i> GST-Yip1p (pRS305)	This laboratory [13]
pRC1245	YCP50 containing <i>YIP1</i> with endogenous 5' and 3' UTR	This study
pRC1578	<i>LEU2</i> CEN <i>GAL<sub>1/10</sub></i> GST-Yip4p (pRS315)	This study
pRC1579	<i>LEU2</i> CEN <i>GAL<sub>1/10</sub></i> GST-Yif1p (pRS315)	This study

at least 30 independent colonies were assayed for  $\beta$ -galactosidase activity.  $\beta$ -Galactosidase activity was determined with the chromogenic substrate X-gal using a Macintosh computer-based imaging analysis with CanoScan N670U using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/ni-image/>)

### 2.3. Co-precipitation experiments

Rab proteins as indicated were expressed as glutathione *S*-transferase (GST) fusion proteins under the control of the *GAL*<sub>1/10</sub> promoter in yeast. These strains contain a plasmid expressing either mal-

tose binding protein (MBP)-tagged Yip1p, Yif1p or Yip4p. The experimental protocol was as described in [13]. Strains used for pull-down experiments were grown overnight in 50 ml of selective medium containing galactose as carbon source (SGal) to an absorbance of  $\sim 0.7$  A<sub>600</sub>. Cells were harvested by centrifugation at 4°C and washed in 1 ml of ice-cold buffer (10 mM Tris pH 7.5, 10 mM Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub>). Cell pellets were resuspended in 100  $\mu$ l of ice cold lysis buffer (20 mM KPi pH 7.5, 80 mM KCl, 1 mM EGTA, 2% glycerol, 0.8% Tween 20) containing protease inhibitors (10 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml pepstatin A) before lysis with glass beads. A total detergent-solubilized extract was generated by incubating lysates

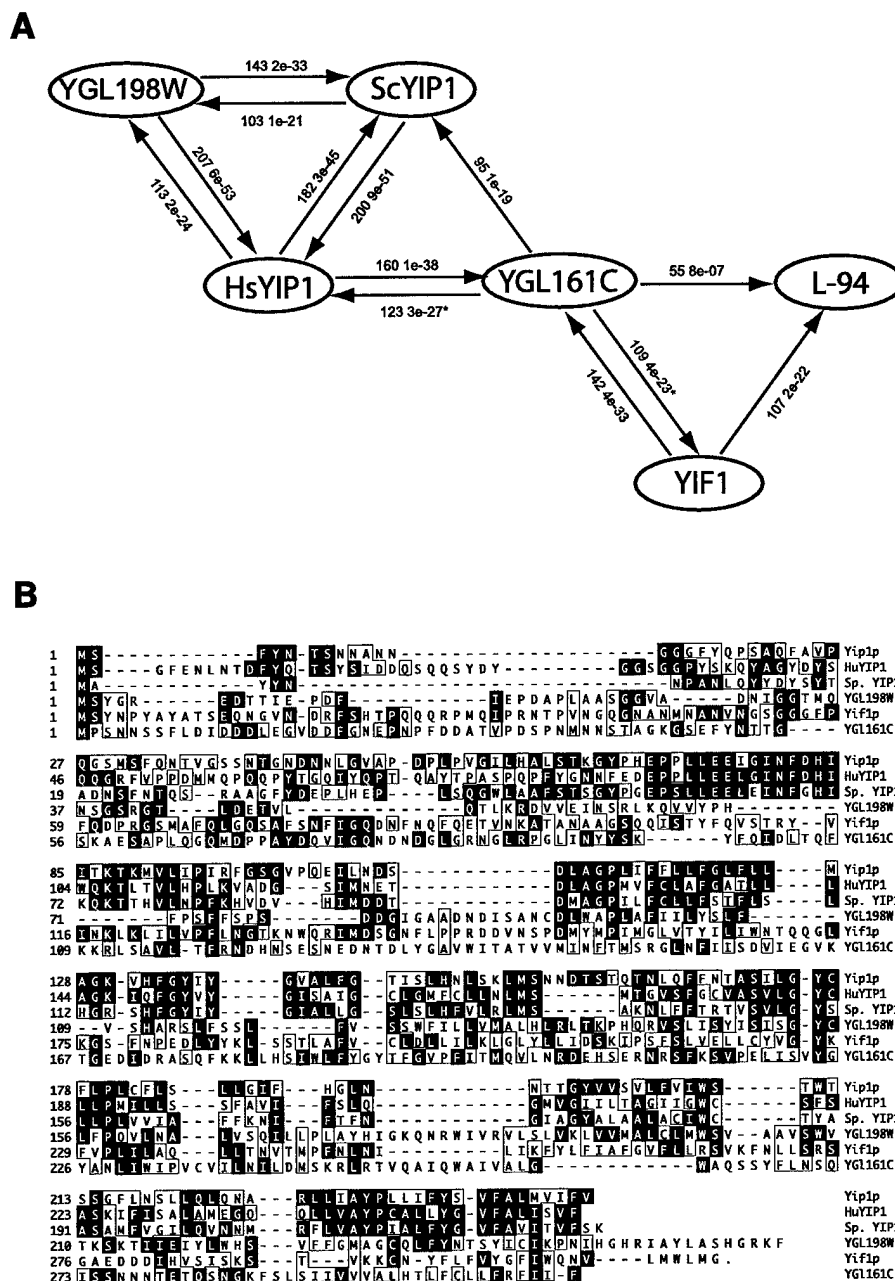


Fig. 1. A: PSI-BLAST identification of related YIP1 sequences. PSI-BLASTP 2.2.1 was performed on each protein sequence indicated. Relationships identified are indicated using lines whose directionality points from the query sequence towards the identified sequence. Analysis was carried out using a threshold value of  $p=0.01$  ( $p$  value = 0.1 indicated with asterisk) and BLOSUM 62 matrix against the non-redundant protein database consisting of 772 993 sequences. The complete set of statistical values for these sequence relationships is given in Table 3. B: Alignment of Yip1p with *S. cerevisiae* and human homologs. Sequence of Yip1p and comparison with full length cDNAs from *S. pombe* (SpYIP1), human YIP1 (HsYIP1), Yif1p and the novel *S. cerevisiae* ORFs YGL198W and YGL161C. The sequences were aligned in MegAlign (DNASTAR) using Clustal analysis [25] with a gap length penalty of 10. Amino acid residues are numbered according to the protein sequence. The shaded residues exactly match the consensus sequence, the boxed residues are standard functional groupings [26] of acidic (DE), basic (HKR), hydrophobic (AFILMPVW), and polar (CGNQSTY) residues. Sequence identity values are given in Table 4.

with an additional 1 ml of lysis buffer for 10 min at 4°C. Detergent-solubilized lysates were cleared by two sequential centrifugation steps in a microfuge for 5 min at 13 000 rpm. Samples were incubated with rocking for 30 min at 4°C with 20  $\mu$ l of amylose resin (New England Biolabs). The bead-bound material was washed four times with lysis buffer. Similar procedures were followed for GST pull-downs except glutathione *S*-Sepharose resin (Pharmacia) was used to isolate the GST-tagged proteins. Proteins were eluted from the beads by boiling in SDS sample buffer. The proteins were analyzed by 10% SDS-PAGE gel electrophoresis and Western blotting with anti-GST antibody to detect the presence of the GST-tagged Rab proteins (for these purposes the anti-green fluorescent protein (GFP) antibody Santa Cruz Cat. No. SC-8334, lot G030 was used, this antibody recognized GST in Western blots with far higher avidity than GFP). Anti-MBP antibody (gift of G.R. Whittaker) was used at 1:6000 to detect MBP-tagged proteins. Secondary alkaline phosphatase-conjugated goat anti-rabbit antibodies (Kirkegaard and Perry Laboratories) were added in blocking buffer, followed by washing and chromogenic blot development with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (both from Bio-Rad) substrates in AP buffer (100 mM Tris pH 9.5, 100 mM NaCl and 5 mM MgCl<sub>2</sub>).

Protein expression under the control of the *GAL1/10* promoter was achieved by subcloning the ORF containing the Rab protein in frame with GST into the vector pRC337. These constructs (Table 2) were linearized with a restriction enzyme and integrated into the genome at the *LEU2* locus. Expression of a GST fusion protein of the correct molecular weight was determined by growing the cells in media containing 2% galactose as a carbon source. The plasmid containing MBP-tagged Yip1p (pRC1047) was constructed using polymerase chain reaction to insert a MBP tag cassette immediately after the initiating methionine in order to express the fusion protein under the control of the endogenous promoter and terminator in the yeast vector pRS426.

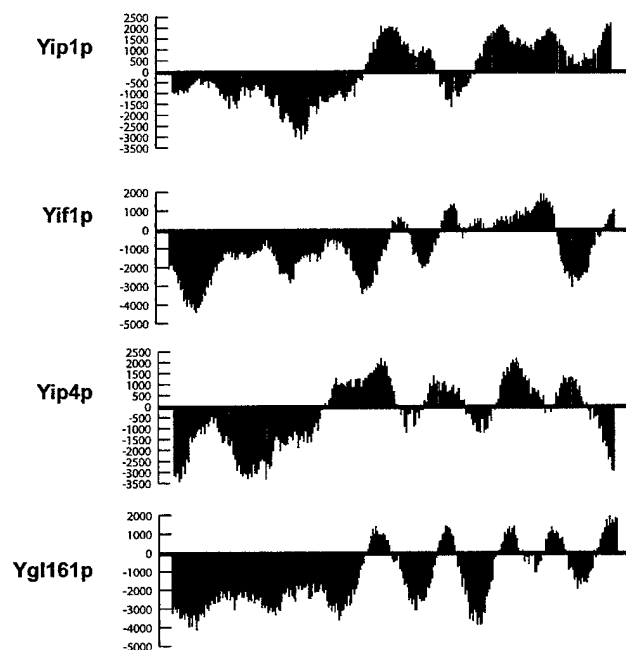


Fig. 2. TMpred plot of Yip1p, Yif1p, Ygl198p and Ygl161p. The TMpred plots for Yip1p, Yif1p, Ygl198p (Yip4p), and Ygl161p (Yip5p) were generated using the program TMpred with a 17 residue minimal and 33 residue maximal length of the hydrophobic part of the transmembrane helix. The TMpred plot shows the relative location of the hydrophobic/hydrophilic segments of the protein. Sequence data indicate a cytoplasmically oriented N-terminus and a hydrophobic C-terminal domain with several potential membrane-spanning/insertion segments.

Table 3  
PSI-BLAST score values amongst YIP1-related proteins

Query	Subject sequence									
	ScYIP1		YGL198W		YGL161C		YIF1		HsYIP1	
	Score (bits)	E value	Score (bits)	E value	Score (bits)	E value	Score (bits)	E value	Score (bits)	E value
ScYIP1	221	3e-57	103	1e-21	34	1.8	36	0.24	200	9e-51
YGL198W	142	2e-33	300	7e-81	NF	NF	NF	NF	207	6e-53
YGL161C	98	1e-19	NF	NF	272	2e-72	109	4e-23	131	7e-30
YIF1	NF	NF	NF	NF	NF	NF	320	7e-87	NF	NF
HsYIP1	182	3e-45	113	2e-24	160	1e-38	33	2.2	296	1e-79
SpYIP1	172	2e-42	114	5e-25	44	0.001	NF	NF	216	9e-56

PSI-BLASTP 2.2.1 was used to identify YIP1-related proteins. Analysis was carried out using a threshold value of  $p = 0.01$  (except for YGL161C for which a  $p$  value = 0.1) and BLOSUM 62 matrix against the non-redundant protein database consisting of 772 993 sequences. Identified sequences converged after five iterations (YGL198W), six iterations (HsYIP1, ScYIP1), seven iterations (YIF1, YGL161C, SpYIP1). NF, not found.

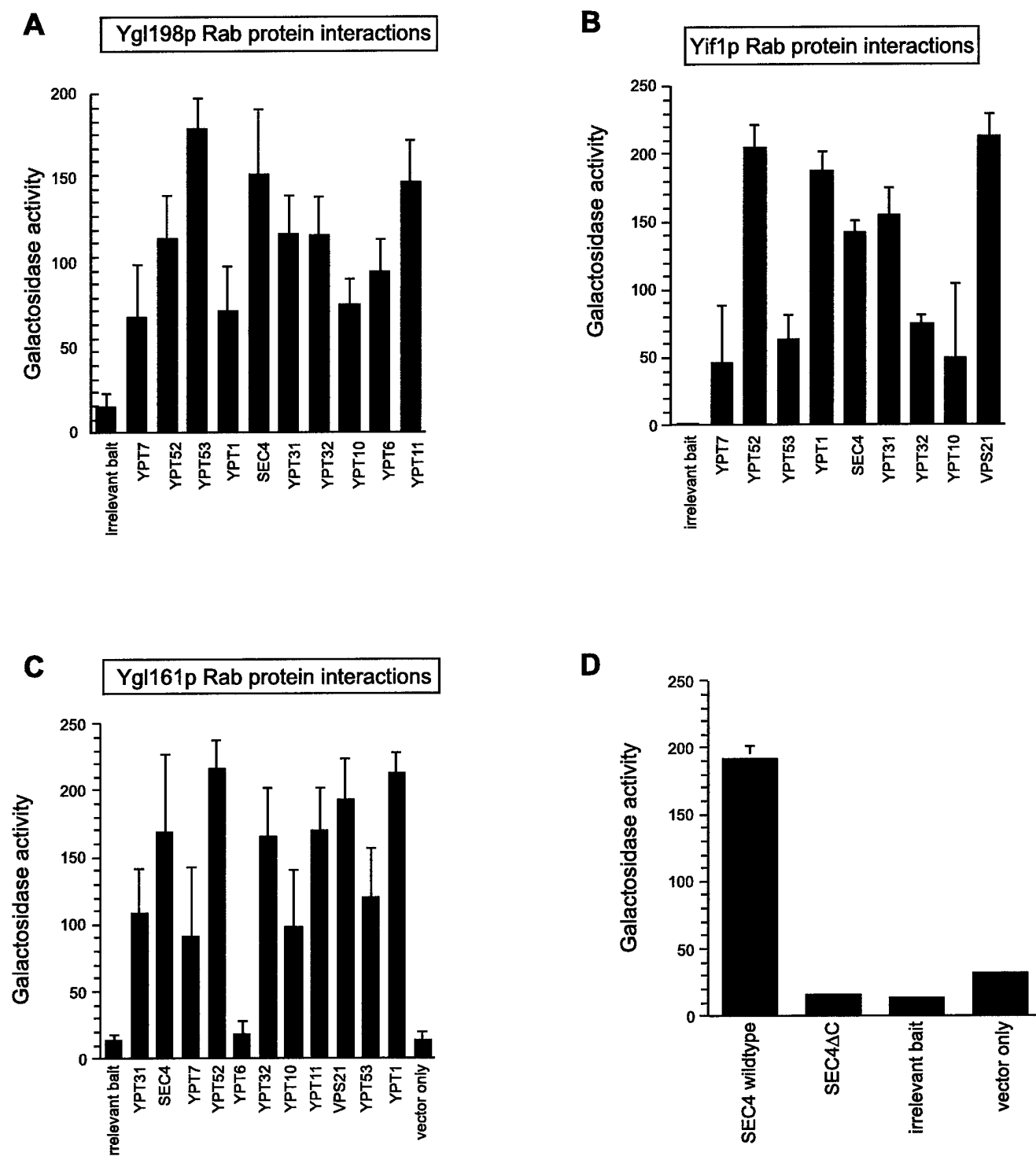


Fig. 3. Y2H interactions of Rab proteins with Yip1p family members. Pairs of constructs were coexpressed in the reporter strain Y190 and  $\beta$ -galactosidase activity (arbitrary units) in the resulting transformants was measured. At least 30 independent transformants were tested for each pair. The Rab protein bait constructs as indicated on the x-axis were tested against prey constructs of Ygl198p (A), Yif1p (B), and Ygl161p (C). D: Ygl161p prey construct tested against the Rab protein Sec4p with and without the C-terminal cysteines. A construct expressing Dss4p (pRC1253) was used as an irrelevant bait control.

### 3. Results and discussion

#### 3.1. A family of Yip1p-related proteins

We used PSI-BLAST [14] with  $p=0.01$  and the BLOSUM62 matrix to identify Yip1p- and HsYip1p-related pro-

teins. This analysis revealed one known ORF (*YIF1*) and one unknown ORF in *S. cerevisiae* (*YGL198W*), unknown ORF SPCC61.04c in *Schizosaccharomyces pombe*, together with numerous expressed sequence tag (EST) fragments from different species, indicating that *YIF1* is part of a gene family conserved among eukaryotes (Fig. 1A and Table 3). Using

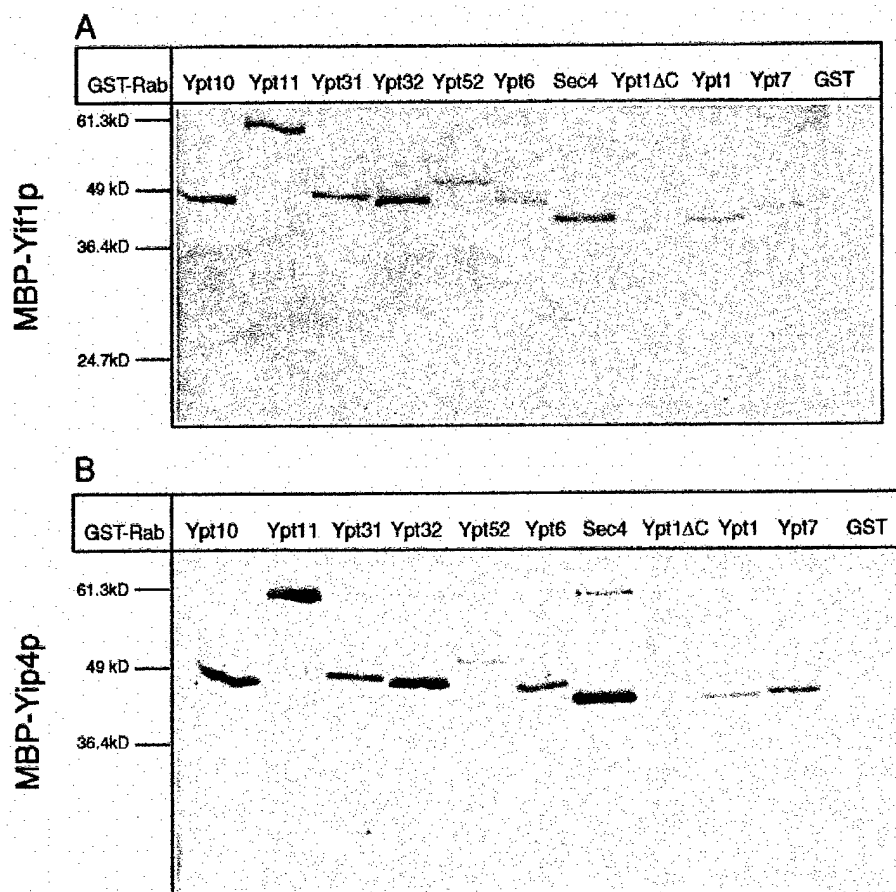


Fig. 4. Co-precipitation of Yip1p-related proteins with Rabs. The panel shows glutathione-resin pull-downs from yeast cells expressing various GST-Rab constructs as indicated. Although the level of expression of proteins in this system is not as high as recombinant expression, it was necessary to use a eukaryotic system due to the dependence of the interaction on correct C-terminal prenylation of the Rab protein. Lysates were prepared from cells expressing either GST alone or various GST-Rab constructs as indicated, together with MBP-tagged Yif1p, or Yip4p. Detergent-solubilized lysates containing 0.5% Tween 20 were incubated with amylose resin for 30 min at 4°C as described in Section 2. After washing, the bead-bound material was subjected to SDS-PAGE electrophoresis and analyzed by Western blotting. Membranes were probed with polyclonal anti-GST (dilution 1:800) to detect the bead-bound GST Rab fusion proteins. Relevant protein marker sizes are indicated. All Rab constructs were under the control of the *GAL<sub>1/10</sub>* promoter and were expressed by inducing with galactose for ~8 h.

Yif1p as the query for a PSI-BLAST search with the same parameters yielded the sequences L1–94 and an unknown ORF, YGL161C, with a convergence after seven iterations. We used the identified ESTs to generate a full length clone for human YIP1 which sequencing revealed was 38.3% identical to that of yeast *YIP1*. This sequence is identical to YIP1A, a human protein that has been reported to localize to endoplasmic reticulum exit sites [15] and also to the smooth muscle cell-associated protein-5 (accession number BAB20270). L1–94 is a partial sequence identified as a putative Rab5-interacting protein from human HeLa cells [16]. Yif1p is a protein previously isolated as a Yip1p interacting factor [17], although its homology to Yip1p was not identified. YGL198W and YGL161C are novel ORFs of unknown function in the *S. cerevisiae* database. The PSI-BLAST score (bits) and *E* values showing the relationships amongst these proteins are shown in Table 3 and a family alignment of the YIP1-related proteins is shown in Fig. 1B. This alignment includes only complete ORFs, L1–94 is not included in the alignment as it is only a partial sequence.

The YIP1-related ORFs identified in our analysis contain significant stretches of hydrophobic residues. We used the TMpred program (<http://www.ch.embnet.org/software/>

TMpred\_form.html) to make a prediction of membrane-spanning regions and their orientation for YIP1, YIF1, YGL198W and YGL161C. The TMpred algorithm is based on the statistical analysis of TMbase, a database of naturally occurring transmembrane proteins using a combination of several weight matrices for scoring [18]. The results of this analysis are shown in Fig. 2. All of these proteins are small (Yip1p 27.1 kDa, Yif1p 35.5 kDa, Ygl198p 29.1 kDa, and Ygl161p 34.8 kDa) with significant hydrophobic segments which potentially span or are inserted into the membrane. All the Yip1p-related proteins share a predicted topology suggesting that they contain two domains. The N-terminus contains the only significant soluble portion of the protein and constitutes one putative domain. The remainder of the protein constitutes the C-terminal domain and contains several potential membrane-spanning segments. The N-terminal domain is oriented towards the cytosol and the C-terminal domain where the hydrophobic segments are located is largely buried in the membrane. Such a topology has been verified experimentally for Yip1p and Yif1p [9,10,17,19]; the results of our sequence analysis would suggest that this topology is also shared by Ygl198p and Ygl161p.

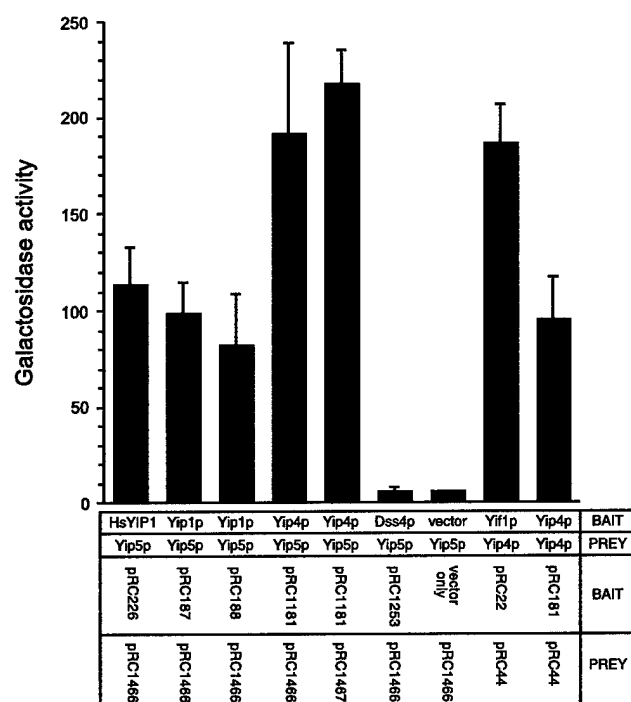


Fig. 5. Yip5p can interact with other YIP1 family members. Pairs of constructs were co-expressed in the reporter strain Y190 and  $\square$ -galactosidase activity in the resulting transformants was measured. At least 30 independent transformants were tested for each pair. The construct pairs are indicated on the x-axis; pRC1466 and pRC1467 are two independent prey constructs which express Ygl161p, and pRC44 is a prey construct expressing Yip4p. pRC187 and pRC188 are two independent bait constructs which express Yip1p; pRC226, pRC181, pRC1253 and pRC22 are bait constructs expressing HsYIP1, Yip4p, Dss4p, and Yif1p respectively. Note the slight variability between two independent constructs expressing identical genes, a common feature of this Y2H system.

### 3.2. Yip1p family members can interact with Rab proteins

To investigate the Yip1p-related proteins further, we examined them for potential Rab protein interactions by both Y2H and biochemical pull-down experiments. We constructed a panel of Y2H constructs containing every Rab protein present in *S. cerevisiae* and tested them against the YIP1-related ORFs identified in Fig. 1A. Y2H analysis (Fig. 3A–C) showed that Yif1p, Ygl198p and Ygl161p are capable of interaction with several Rab proteins. In general we found weaker interactions with the Rab proteins Ypt6p and Ypt7p although these constructs still retained the ability to interact with yeast Rab-GDI in this system (data not shown). These data reveal that YIP1-related proteins are capable of binding to determinants shared by many Rab proteins. We have demonstrated this for Yif1p and two novel ORFs, YGL198W and YGL161C. In addition to Rab interactions, our analysis suggests these proteins share a common overall domain topology with a significant hydrophilic N-terminal segment that is cytoplasmically oriented and a largely hydrophobic C-terminal domain (Fig. 2). ORFs named *YIP2* (also termed *YOP1* [10]) and *YIP3* (also termed *PRA1* [20]) are already present in databases, however it is important to note that these ORFs are unrelated in primary sequence to Yip1p. By analogy with Yip1p and to avoid confusion, we suggest that the ORF YGL198W be named Yip4p (Ypt-interacting protein 4) and YGL161C be named Yip5p (Ypt-interacting protein 5).

A common feature of Rab proteins is the prenylation on two C-terminal cysteine residues by the enzyme geranylgeranyl transferase II [5]. To assess the contribution of this post-translational modification to YIP1 family member interaction we generated a Rab construct lacking its C-terminal cysteines. We chose Sec4p as the representative Rab protein as it interacts well with all the YIP1 family members tested. Y2H experiments, shown in Fig. 3D, demonstrated that interaction of Sec4p with Yip5p was completely dependent on its C-terminal cysteines and presumably on correct post-translational modification of the protein. Biochemical experiments (see below) demonstrated that Rab proteins also require prenylation for stable association with Yif1p and Yip4p.

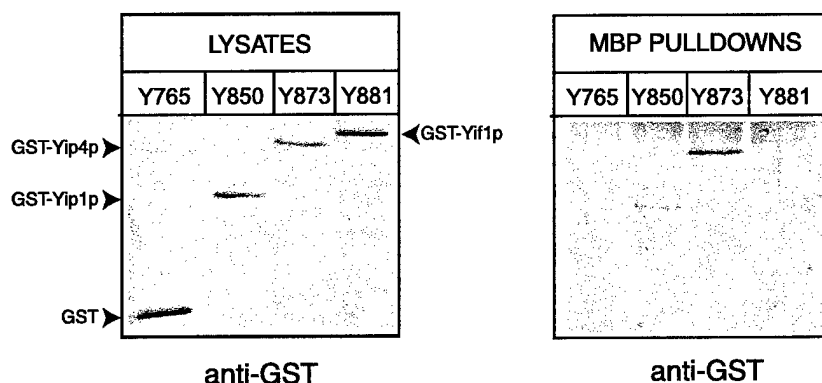
### 3.3. Interaction of Yif1p and Ygl198p with Rab proteins in cellular lysates

To verify the Y2H interactions of YIP1 family members with Rab proteins with an independent technique, we made GST fusions of all yeast Rab proteins. These proteins were expressed under the control of the galactose promoter in yeast, where they would be expected to be correctly post-translationally modified and expressed in cells grown in media with galactose as a carbon source. Expression of a GST fusion of the expected size could be observed for each Rab protein (data not shown). We tested the GST-Rab protein fusions for biochemical interaction by co-precipitation with Yif1p and Yip4p. Yif1p and Yip4p were tagged with an N-terminal MBP fusion and expressed from endogenous promoters. The cellular lysates were incubated with amylose resin for 30 min at 4°C to pull down the MBP-Yif1p or MBP-Yip4p protein. After extensive washing, the bead-bound material was analyzed by SDS-PAGE and Western blotting. The Western blots were probed with anti-GST polyclonal antibody to detect any associated Rab proteins. The results of this analysis are shown in Fig. 4. MBP-Yif1p and Yip4p did not co-precipitate with GST alone, and neither with a Ypt1p construct lacking its C-terminal cysteines which are the sites of prenylation. Both MBP-Yif1p and Yip4p were able to interact with several different Rab proteins. These results parallel the data obtained in the two-hybrid assay and show that Yip1p-related proteins interact with diverse Rab proteins in cellular lysates. Do Rab proteins show different affinities for YIP1 proteins? A precise answer is beyond the scope of this study, however our data (Fig. 4) show that the amount of protein that is precipitated varies between individual Rab proteins. As the expression level of the Rab proteins does not vary significantly this suggests that Rab proteins may have preferences for the YIP1 family member with which they associate. This suggestion must be taken with caution however, as these experiments have utilized tagged proteins which may also influence the observed strength of interaction. If YIP1 family members display differential affinities for each Rab protein this would imply that prenylation, although necessary, is not the sole determinant for interaction.

### 3.4. Interactions amongst Yip1p family members

Yif1p was originally identified as a Yip1p binding partner although its identity as a YIP1-related sequence has not previously been identified [17]. In addition, several Y2H high-throughput screens have identified a plethora of Yip1p-interacting factors amongst which are included YGL198W (*YIP4*) and YGL161C (*YIP5*) [21–23]. These data suggest that Yip1p

## A. MBP Pulldowns (MBP-Yip4)



## B. GST Pulldowns (GST-Yip1)

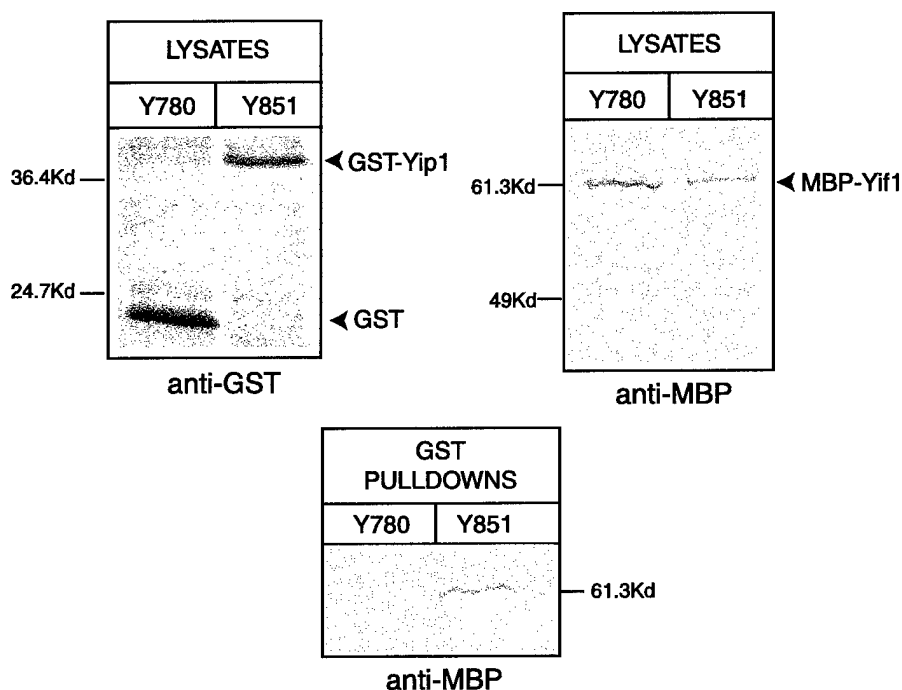
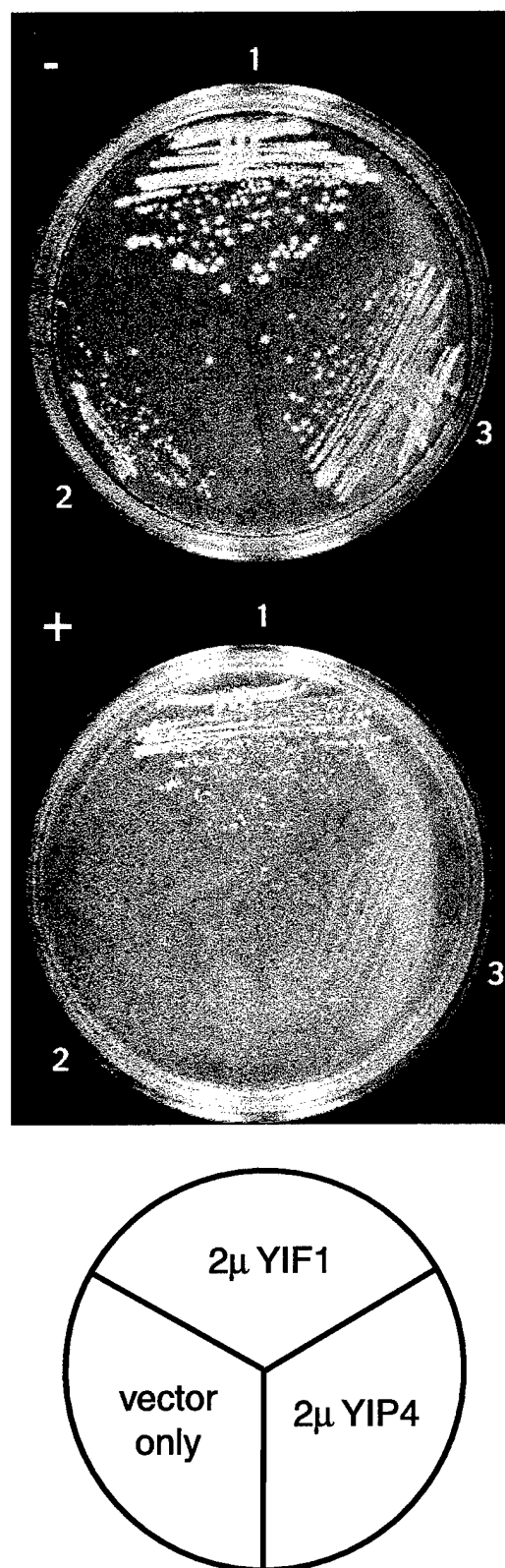


Fig. 6. Biochemical analysis of Yip1p interactions with the Yip1p family members Yif1p and Yip4p. Lysates were prepared from yeast cells expressing (A) GST alone, GST-Yip1p, GST-Yip4p or GST-Yif1p together with MBP-Yip4p (B) GST alone or GST-Yip1p together with MBP-Yif1p (B). Detergent-solubilized total cell lysates were incubated with GST beads (A) or amylose resin (B) for 30 min at 4°C as described in Section 2. After washing, the bead-bound material was subjected to SDS-PAGE electrophoresis and analyzed by Western blotting. Membranes were probed with polyclonal anti-GST (1:800) to detect GST-Yip1p (A) and polyclonal anti-MBP (1:6000) to detect MBP-Yif1p (B). Relevant protein marker sizes are indicated. GST-Yip1p, GST-Yip4p and GST-Yif1p but not GST alone could be detected after MBP-Yip4p pull-downs. MBP-Yif1p could be detected in RCY851 but not RCY780 after glutathione resin pull-downs.

has an ability to physically associate with other YIP1-related sequences. We wished to examine whether YIP1 family members in general share the ability to physically associate amongst themselves. We decided to test these interactions biochemically in deliberate pairwise combinations in both Y2H and biochemical co-precipitation experiments. We chose *YIP5* to test interactions in the Y2H system. The results of this analysis are shown in Fig. 5. Yip5p interacted very strongly with Yip4p and less strongly with Yip1p or the mammalian sequence HsYIP1. Yip4p was also able to self-associate with

an interaction level comparable to its interaction with Yip1p. No interactions were observed with an irrelevant plasmid and the Yip5p plasmid showed no autoactivation. As expected, Yip1p and Yif1p also showed strong interactions in the Y2H system.

For the co-precipitation experiments, Yip1p, Yip4p and Yif1p were tagged with GST, Yip4p and Yif1p were tagged with MBP. GST alone was used as a control. An amylose resin pull-down from detergent-solubilized lysates of cells expressing MBP-Yip4p together with either GST alone, GST-



Yip1p, GST-Yip4p or GST-tagged Yif1p revealed that Yip1p, Yif1p and Yip4p could be specifically co-precipitated with Yip4p (Fig. 6A). For Yif1p, we performed the reverse experiment, the GST alone or GST-Yip1p constructs were expressed in cells together with MBP-Yif1p and isolated from detergent solubilized extracts with glutathione agarose. The bead-bound

Fig. 7. High-copy plasmids containing the YIP1-related sequence *YIF1* can bypass the requirement for *YIP1*. Cells bearing their only copy of *YIP1* on plasmid containing the counter-selectable marker *URA3* were tested for ability to grow on 5-FOA after transformation with the *YIP1*-related ORFs *YIF1* and *YIP4*. Colonies transformed with multi-copy vectors containing (1) *YIF1*, (2) no insert control, or (3) *YIP4* (YGL198W) were tested for growth on synthetic media with and without 5-FOA to select against retention of the *YIP1* plasmid. Only cells containing multi-copy *YIF1* can survive the loss of the *YIP1*-containing plasmid on 5FOA.

material was probed for associated MBP-Yif1p with an anti-MBP antibody (Fig. 6B). This experiment demonstrated that Yif1p can physically interact with Yip1p, a result which confirms previous findings [17] and demonstrates that the tag used for our experiments does not interfere with protein–protein interactions. Our data confirm and extend the Y2H observations identified in high-throughput screens for Yip1p and suggest that the ability for YIP1 family members to interact amongst themselves is a common feature. Clearly, further experiments are required to ascertain the precise oligomeric nature of these YIP1 family member complexes and determine if the family members have particular preferences for association amongst themselves.

### 3.5. Overlapping functions of *Yip1p* family members

Our results demonstrate that YIP1 family members share a common domain topology, bind to Rab proteins in a prenylation-dependent manner and can physically associate amongst themselves. To what extent do the YIP1-related proteins have distinct and overlapping functions? We can begin to answer some of these questions through manipulation of the relevant genes in a genetically tractable organism such as yeast. One of the most stringent tests of function is to ask if one gene can functionally substitute for the deletion of the other. *YIP1* is an essential gene [9] so we tested *YIP4* and *YIF1* for the ability to complement *YIP1* function by asking if these genes could overcome the loss of *YIP1* when expressed from a multicopy plasmid. For this experiment, a strain was generated where the genomic copy of *YIP1* was deleted and viability was maintained by the inclusion of an episomal plasmid containing *YIP1* with a counter-selectable marker, *URA3*. The strain was transformed with a multi-copy plasmid encoding either *YIF1* or *YIP4* and plated on media containing fluoroarotic acid (5-FOA) to select against the *YIP1* gene. Remarkably, *YIF1* overexpression can overcome the loss of *YIP1*; however, *YIP4* was unable to do so (Fig. 7). There are several possible explanations for this result. The overexpression of a gene can suppress defects in other gene products by providing a similar function to that of the absent gene, by providing an alternative pathway or by bypassing the requirement for the absent gene if the suppressor gene lies downstream in the pathway. The fact that *YIF1* can substitute for the absence of *YIP1* indicates that it performs a similar function, further strengthening the suggestion that the YIP1 family may have shared functions and interacting partners. *YIP4* cannot substitute for the loss of *YIP1* indicating that this gene may function upstream of *YIP1* or may act on a different pathway even though these two genes share several potential interacting partners.

Groupings of small membrane proteins with significant hydrophobic segments such as those of the YIP1 family are difficult to establish by conventional means such as BLAST

Table 4  
Sequence distances amongst YIP1-related proteins

	1	2	3	4	5	6	
1		30.2	38.3	12.9	14.9	13.3	Yip1p
2	64.3		38.8	12.1	13.2	12.5	HsYIP1
3	59.3	58.2		14.5	13.2	12.8	SpYIP1
4	82.8	83.9	82.5		13.8	11.5	YGL198W
5	84.7	84.5	85.9	79.1		11.9	Yif1p
6	84.2	84.5	85.0	88.2	86.9		YGL161C

The sequence distance table shows the calculated divergence and similarity of each pair of sequences aligned by the Clustal method as outlined in Fig. 1

algorithm searches and must be supported by additional experimental criteria. We propose that for the YIP1-related family, these criteria are: (i) a topology that includes a significant N-terminal hydrophilic domain that faces the cytosol with an hydrophobic C-terminal domain, (ii) the ability to interact with Rab proteins in a manner dependent on C-terminal prenylation, and (iii) the ability to associate with other members of the YIP1 family. We have demonstrated the unknown ORFs YGL198W (*YIP4*) and YGL161C (*YIP5*) are also Rab-interacting factors and bona fide Yip1p homologs even though they share very little sequence similarity (Table 4). The putative Rab5-interacting protein L1–94 shares two of these criteria [16] and we predict it also to be a member of the YIP1 protein family.

What is the cellular role played by Yip1p-related proteins? One possibility is that they serve as membrane proteins which aid in the recruitment of Rab proteins from the cytosol onto membranes, enabling Rab proteins to be correctly localized and used for many rounds of vesicle transport. Our data suggest that YIP1-related proteins are potential membrane counterparts to Rab-GDI. Similarly to Rab-GDI, they are biochemically capable of interacting with different Rab proteins in a manner dependent on the C-terminal prenylation, perhaps indicating that they can compete with Rab-GDI for Rab protein association. Although there is a plethora of evidence indicating that Rab proteins act downstream of vesicle budding, it is becoming apparent that Rab proteins may also play critical roles in vesicle biogenesis [2]. One rationalization for this may be that a functional vesicle must be equipped with the membrane components required for tasks at a later stage. V-SNAREs, for example, are required for fusion with the acceptor membrane, so these proteins must be included into nascent vesicles with high fidelity. Rab proteins too must be incorporated into the transport vesicle, implying a link between the Rab recruitment machinery and vesicle biogenesis. In support of this idea, Yip1p has been observed to interact with the SNARE protein *TLG1* [23] and we have recently obtained information that Yip1p will interact with the v-SNARE *SNC2* in the Y2H system (unpublished data). Although these data are preliminary and we do not know how far this extends to other YIP1 family members, it is tempting to speculate that there is a functional significance to this interaction. Further strengthening this suggestion is the finding that Yip1p and Yif1p have been observed to be

selectively packaged into COPII vesicles in vitro [24], perhaps providing a link between YIP1 family members, Rab proteins and the vesicle biogenesis machinery. Clearly much remains to be understood about these important and intriguing membrane proteins.

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